UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF MASSACHUSETTS
CENTRAL DIVISION

ABBOTT BIOTECHNOLOGY LTD., and ABBOTT LABORATORIES Plaintiffs, v. CENTOCOR ORTHO BIOTECH INC., Defendant.	CIVIL ACTION NO. 09-40089-FDS **DEMAND FOR JURY TRIAL**

ABBOTT'S SECOND AMENDED COMPLAINT

Plaintiff Abbott Biotechnology Limited ("ABL" or "Plaintiff") and Abbott Laboratories ("Abbott" or "Plaintiff") hereby bring this Second Amended Complaint for patent infringement against Defendant Centocor Ortho Biotech Inc. ("Centocor" or "Defendant"). Pursuant to Rule 15(a)(2) of the Federal Rules of Civil Procedure, Centocor has given its written consent to this amendment.

NATURE OF ACTION

1. This is an action for patent infringement by and against the above-named Defendant.

PARTIES

2. Plaintiff ABL is a corporation organized under the laws of Bermuda, with a place of business at Clarendon House, 2 Church Street, Hamilton HM11, Bermuda.

3. Through intermediate organizations, ABL is owned by Plaintiff Abbott Laboratories, an Illinois corporation which conducts business in this District directly as well as through its

subsidiary Abbott Bioresearch Center, Inc. ("ABC"), located in Worcester, Massachusetts (as more fully described below).

4. Defendant Centocor is a corporation organized under the laws of the Commonwealth of Pennsylvania, with a principal place of business at Ridgeview Drive, Horsham, Pennsylvania. Within the meaning of 28 U.S.C. § 1391(c), it resides in this District by reason of having continuous and systematic contacts with this District, including the marketing and sales of health products in Massachusetts, such as the drug marketed and sold as "REMICADE," which enjoyed U.S. sales of approximately $2.8 billion in 2008.

JURISDICTION AND VENUE

5. This is an action for patent infringement arising under the Patent Laws of the United States, 35 U.S.C. § 271 et seq.

6. This Court has subject matter jurisdiction over this action under 28 U.S.C. §§ 1331 and 1338.

7. As Centocor's Answer has admitted, Defendant has been engaged in efforts to meet the regulatory requirements of the United States Food and Drug Administration ("FDA") for marketing, distributing, offering for sale and/or selling SIMPONI™, an anti-TNF alpha therapy product that Defendant has sought FDA approval for with respect to the treatment of rheumatoid arthritis. *See* www.simponi.com (April 28, 2009).

8. As Centocor's Answer has admitted, on or about April 24, 2009, Defendant obtained FDA approval to market SIMPONI™ in the United States for use in combination with methotrexate as a treatment for rheumatoid arthritis. Defendant has been manufacturing, distributing, using, offering for sale, selling, and/or importing into the United States the

SIMPONI™ therapy product, to be prescribed and used for the treatment, *inter alia*, of rheumatoid arthritis in combination with methotrexate.

9. On May 29, 2007, the United States Patent & Trademark Office ("USPTO") duly and legally issued United States Patent No. 7,223,394 (the "'394 Patent"), entitled "Human Antibodies that Bind Human TNF alpha," to the listed inventors Jochen G. Salfeld of North Grafton, Massachusetts, and other co-inventors in Massachusetts (and other locations). Dr. Salfeld and one or more of his co-inventors are current employees at the earlier-mentioned Abbott Bioresearch Center, Inc. ("ABC"), located at 100 Research Drive in Worcester, Massachusetts, where they carried out the research leading to the '394 patent. The '394 Patent has claims relating to methods for treating a subject suffering from rheumatoid arthritis by coadministering both a human antibody and methotrexate. The antibody in the claimed methods has particular affinity for and neutralizes human TNF alpha. Plaintiff ABL is the assignee and owner of the '394 Patent, a copy of which is attached as Exhibit A. Plaintiffs' product HUMIRA® is a fully human TNF alpha neutralizing antibody whose use together with methotrexate to treat rheumatoid arthritis is covered by the '394 patent. Since its launch in 2002, HUMIRA® has benefitted hundreds of thousands of patients worldwide.

10. On June 2, 2009, the USPTO duly and legally issued U.S. Patent No. 7,541,031 (the "'031 Patent"), entitled "Methods for Treating Rheumatoid Arthritis using Human Antibodies that Bind Human TNF," with listed inventors Jochen G. Salfeld of North Grafton, Massachusetts, and other co-inventors in Massachusetts (and other locations). As noted above, Dr. Salfeld and one or more of his co-inventors are current employees at ABC, where they carried out the research leading to the '031 Patent. The '031 Patent is a continuation application of the above-described '394 patent, and has claims relating to a method for treating a subject

suffering from rheumatoid arthritis by administering a human antibody that binds TNFand methotrexate. Plaintiff ABL is the assignee and owner of the '031 Patent, a copy of which is attached as Exhibit B.

11. Plaintiff Abbott Laboratories has an exclusive license under the '394 and '031 Patents as a result of its October 7, 2010, license agreement with Plaintiff ABL.

12. Defendant is infringing Plaintiffs' '394 Patent by virtue of the offer for sale and sale in the United States of its SIMPONI™ product with the rheumatoid-arthritis indication discussed above, including by inducing infringement. By way of example, Centocor's prescribing information for SIMPONI™ states that "[f]or patients with rheumatoid arthritis (RA), SIMPONI should be given in combination with methotrexate."

13. Defendant is infringing Plaintiffs' '031 Patent by virtue of the offer for sale and sale in the United States of its SIMPONI™ product with the rheumatoid-arthritis indication discussed above, including by inducing infringement. By way of example, Centocor's prescribing information for SIMPONI™ states that "[f]or patients with rheumatoid arthritis (RA), SIMPONI should be given in combination with methotrexate."

14. Venue is proper in this judicial district under 28 U.S.C. §§ 1391(b), (c) and/or 1400(b), as *inter alia* the Defendant is subject to personal jurisdiction in this District.

COUNT I

Infringement of the '394 Patent

15. Plaintiffs incorporate by reference paragraphs 1-14 as if fully set forth herein.

16. As set forth above, an actual controversy now exists between the parties that Defendant's SIMPONI™ product, when co-administered with methotrexate to treat rheumatoid arthritis (per Centocor's indication), is infringing Plaintiffs' '394 Patent.

17. As set forth above, by making meaningful preparations to market, make, use, sell, offer for sale, and/or import SIMPONI™ into the United States, with the rheumatoid-arthritis indication discussed above, and having now recently obtained FDA approval and making, using, selling, offering for sale, and/or importing SIMPONI™, Defendant Centocor is engaged in activities that infringe the '394 Patent, including by inducing infringement under 35 U.S.C. § 271(b).

18. Defendant is aware of the '394 Patent and its infringement is deliberate, willful, and in reckless disregard of Plaintiffs' patent rights.

19. Plaintiffs have been and will continue to be injured by the infringing activities of Defendant.

COUNT II

Infringement of the '031 Patent

20. Plaintiff incorporates by reference paragraphs 1-19 as if fully set forth herein.

21. As set forth above, an actual controversy now exists between the parties that Defendant's SIMPONI™ product, when co-administered with methotrexate to treat rheumatoid arthritis (per Centocor's indication), is infringing Plaintiffs' '031 Patent.

22. As set forth above, by making meaningful preparations to market, make, use, sell, offer for sale, and/or import SIMPONI™ into the United States, with the rheumatoid-arthritis indication discussed above, and having now recently obtained FDA approval and making, using, selling, offering for sale, and/or importing SIMPONI™, Defendant Centocor is engaged in activities that infringe the '031 Patent, including by inducing infringement under 35 U.S.C. § 271(b).

23. Defendant is aware of the '031 Patent and its infringement is deliberate, willful, and in reckless disregard of Plaintiffs' patent rights.

24. Plaintiffs have been and will continue to be injured by the infringing activities of Defendant.

PRAYER FOR RELIEF

WHEREFORE, Plaintiffs ABL and Abbott Laboratories respectfully request the following relief:

(a) a finding that Defendant infringes the '394 Patent and a final judgment incorporating same;

(b) a finding that Defendant infringes the '031 Patent and a final judgment incorporating same;

(c) entry of preliminary and/or permanent equitable relief, including but not limited to an order preliminary and/or permanent injunction that enjoins Defendant and any of its officers, agents, employees, assigns, representatives, privies, successors, and those acting in concert or participation with them from infringing and/or inducing infringement of the '394 Patent and the '031 Patent;

(d) an award of damages sufficient to compensate Plaintiffs for infringement of the '394 Patent by Defendant, together with prejudgment and post-judgment interest;

(e) an award of damages sufficient to compensate Plaintiffs for infringement of the '031 Patent by Defendant, together with prejudgment and post-judgment interest;

(f) entry of an order compelling Defendant to compensate Plaintiffs for any ongoing and/or future infringement of the '394 Patent, in an amount and under terms appropriate under the circumstances;

(g) entry of an order compelling Defendant to compensate Plaintiffs for any ongoing and/or future infringement of the '031 Patent, in an amount and under terms appropriate under the circumstances;

(h) a declaration or order finding that Defendant's infringement is willful and/or an order increasing damages under 35 U.S.C. § 284;

(i) a judgment holding that this is an exceptional case under 35 U.S.C. § 285 and awarding Plaintiffs their reasonable attorneys fees, costs, and expenses;

(j) such other relief deemed just and proper.

JURY DEMAND

Under Rule 38 of the Federal Rules of Civil Procedure, Plaintiffs hereby demand trial by jury of all issues so triable by a jury in this action.

DATED: October 12, 2010

Respectfully submitted,

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CERTIFICATE OF SERVICE

I hereby certify that this document filed through the ECF system will be sent electronically to the registered participants as identified on the Notice of Electronic Filing (NEF) and paper copies will be sent to those indicated as non-registered participants on October 12, 2010.

/s/ Michael P. Angelini
Michael P. Angelini

EXHIBIT A

US007223394B2

(12) **United States Patent**
Salfeld et al.

(10) **Patent No.: US 7,223,394 B2**
(45) **Date of Patent: *May 29, 2007**

(54) **HUMAN ANTIBODIES THAT BIND HUMAN TNFα**

(75) Inventors: **Jochen G. Salfeld**, North Grafton, MA (US); **Deborah J. Allen**, Cambridge (GB); **Hendricus R. J. M. Hoogenboom**, Maastricht (BE); **Zehra Kaymakcalan**, Westboro, MA (US); **Boris Labkovsky**, Framingham, MA (US); **John A. Mankovich**, Andover, MA (US); **Brian T. McGuinness**, Comberton (GB); **Andrew J. Roberts**, Cambridge (GB); **Paul Sakorafas**, Newton, MA (US); **David Schoenhaut**, Garfield, NJ (US); **Tristan J. Vaughan**, Impington (GB); **Michael White**, Framingham, MA (US); **Alison J. Wilton**, Cambridge (GB)

(73) Assignee: **Abbott Biotechnology Ltd**, Hamilton (BM)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/801,185**

(22) Filed: **Mar. 7, 2001**

(65) **Prior Publication Data**

US 2003/0092059 A1 May 15, 2003

Related U.S. Application Data

(63) Continuation of application No. 09/125,098, filed on Mar. 16, 1999, now Pat. No. 6,258,562.

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/142.1**; 424/145.1; 424/158.1; 530/388.15; 530/388.23; 530/389.2

(58) **Field of Classification Search** 424/142.1, 424/145.1, 158.1; 530/388.15, 388.23, 389.2
See application file for complete search history.

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(Continued)

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(57) **ABSTRACT**

Human antibodies, preferably recombinant human antibodies, that specifically bind to human tumor necrosis factor a (hTNFα) are disclosed. These antibodies have high affinity for hTNFα (e.g., K_d 10^{-8} M or less), a slow off rate for hTNFα dissociation (e.g., K_{off} 10^{-3} sec^{-1} or less) and neutralize hTNFα activity in vitro and in vivo. An antibody of the invention can be a full-length antibody or an antigen-binding portion thereof. The antibodies, or antibody portions, of the invention are useful for detecting hTNFα and for inhibiting hTNFα activity, e.g., in a human subject suffering from a disorder in which hTNFα activity is detrimental. Nucleic acids, vectors and host cells for expressing the recombinant human antibodies of the invention, and methods of synthesizing the recombinant human antibodies, are also encompassed by the invention.

10 Claims, 11 Drawing Sheets

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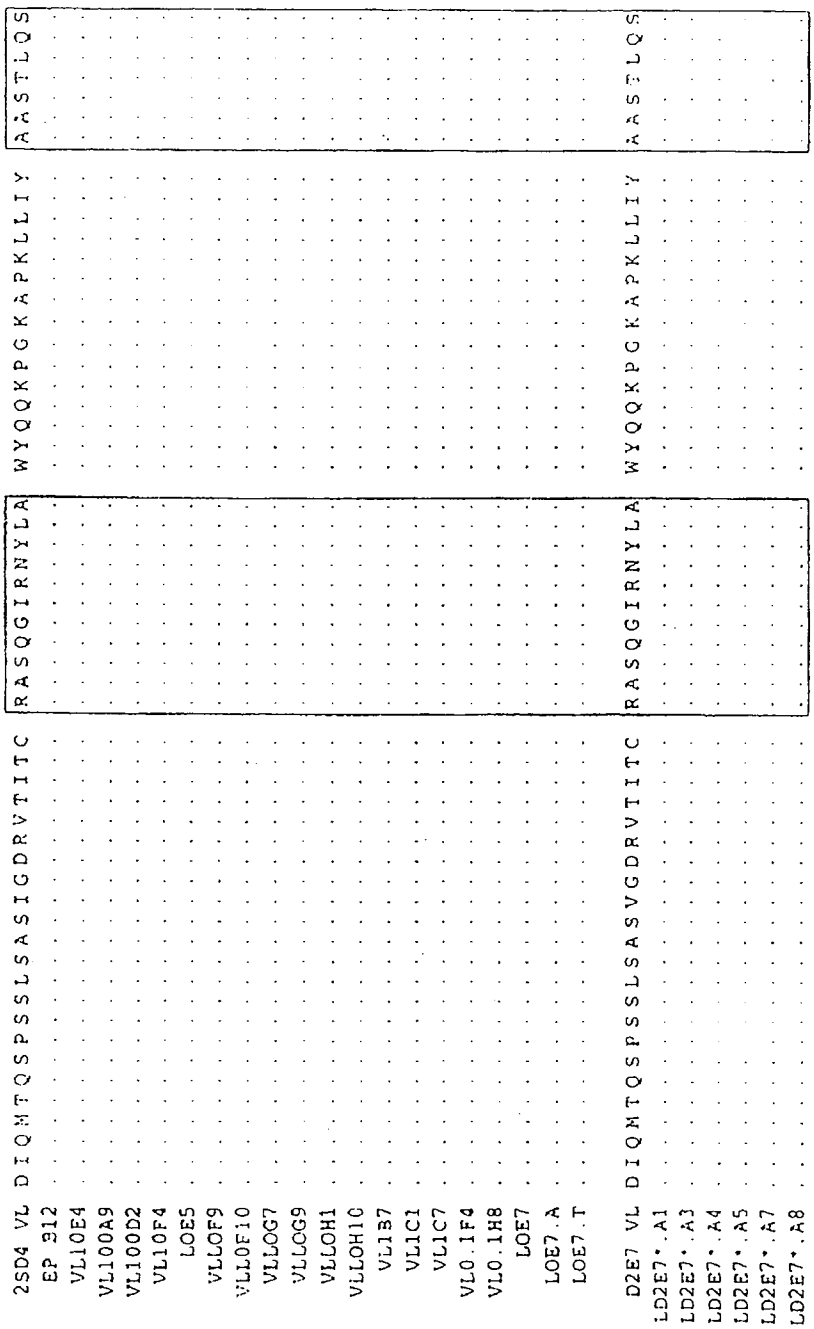
CDR L1
CDR L2
2SD4 VL DIQMTQSPSSLSASIGDRVTITC RASQGIRNYLA WYQQKPGKAPKLLIY AASTLQS
EP 312 . . . . . .
VL10E4 . . . . . .
VL100A9 . . . . . .
VL100D2 . . . . . .
VL10F4 . . . . . .
LOE5 . . . . . .
VLLOF9 . . . . . .
VLLOF10 . . . . . .
VLLOG7 . . . . . .
VLLOG9 . . . . . .
VLLOH1 . . . . . .
VLLOH10 . . . . . .
VL1B7 . . . . . .
VL1C1 . . . . . .
VL1C7 . . . . . .
VL0.1F4 . . . . . .
VL0.1H8 . . . . . .
LOE7 . . . . . .
LOE7.A . . . . . .
LOE7.T . . . . . .
D2E7 VL DIQMTQSPSSLSASVGDRVTITC RASQGIRNYLA WYQQKPGKAPKLLIY AASTLQS
LD2E7*.A1 . . . . . .
LD2E7*.A3 . . . . . .
LD2E7*.A4 . . . . . .
LD2E7*.A5 . . . . . .
LD2E7*.A7 . . . . . .
LD2E7*.A8 . . . . . .

Figure 1A

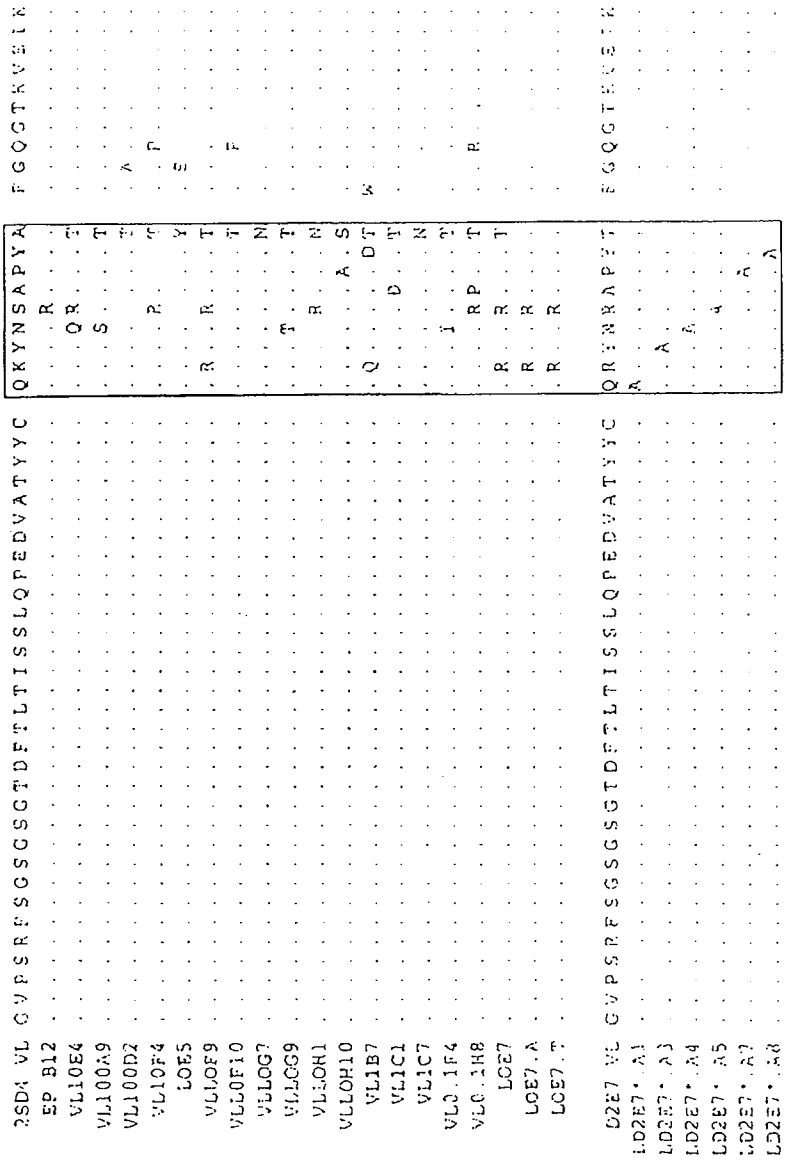

Figura 1B

CDR H1
CDR H2
2SD4 VH QVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH WVRQAPGKGLEWVS AITWNSGHIDYADSVEG
VH1B11
VH1D8
VH1A11
VH1B12
VH1-D2
VH1E4
VH1F6
VH1G1
JC-H2
VH1-D2.N
VH1-D2.Y
D2E7 VH EVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH WVRQATGKGLEWVS LITWNSGHIEYADSVEG
HD2E7*.A1
HD2E7*.A2
HD2E7*.A3
HD2E7*.A4
HD2E7*.A5
HD2E7*.A6
HD2E7*.A7
HD2E7*.A8
HD2E7*.A9

Figure 2A

CDR H3
1 2 3 4 5 6 7 8 9 10 11 12
2SD4 VH R F A V S R D N A K N T A Y L Q M N S L R P E D T A V Y Y C T K W G Q G T L V T V S S
VH1B11
VH1D8
VH1A11
VH1B12
VH1-D2
VH1E4
VH1F6
VH1G1
3C-H2
VH1-D2.N
VH1-D2.Y
D2E7 VH R F T I S R D N A K N S L Y L Q M N S L R A E D T A V Y Y C A K V S Y L S T A S S L D Y W G Q G T L V T V S S
HD2E7*.A1
HD2E7*.A2
HD2E7*.A3
HD2E7*.A4
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HD2E7*.A6
HD2E7*.A7
HD2E7*.A8
HD2E7*.A9

Figure 2B

FIG. 3

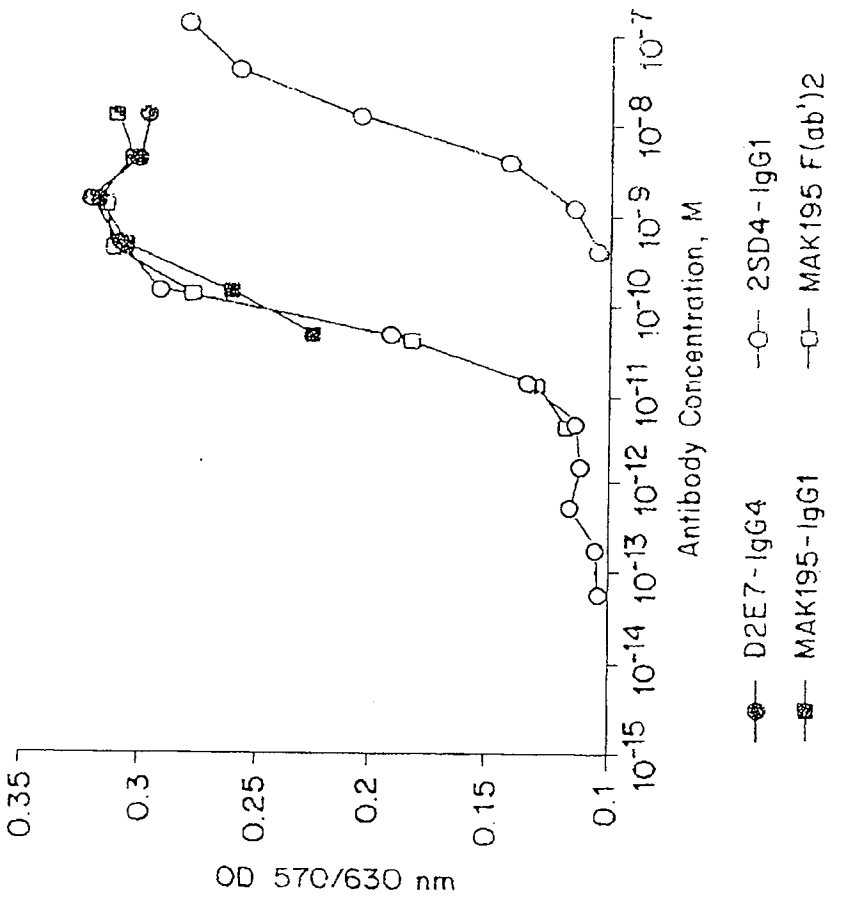

OD 570/630 nm
0.35
0.3
0.25
0.2
0.15
0.1
10^{-15} 10^{-14} 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
Antibody Concentration, M
D2E7-IgG4
2SD4-IgG1
MAK195-IgG1
MAK195 F(ab')2

FIG. 4

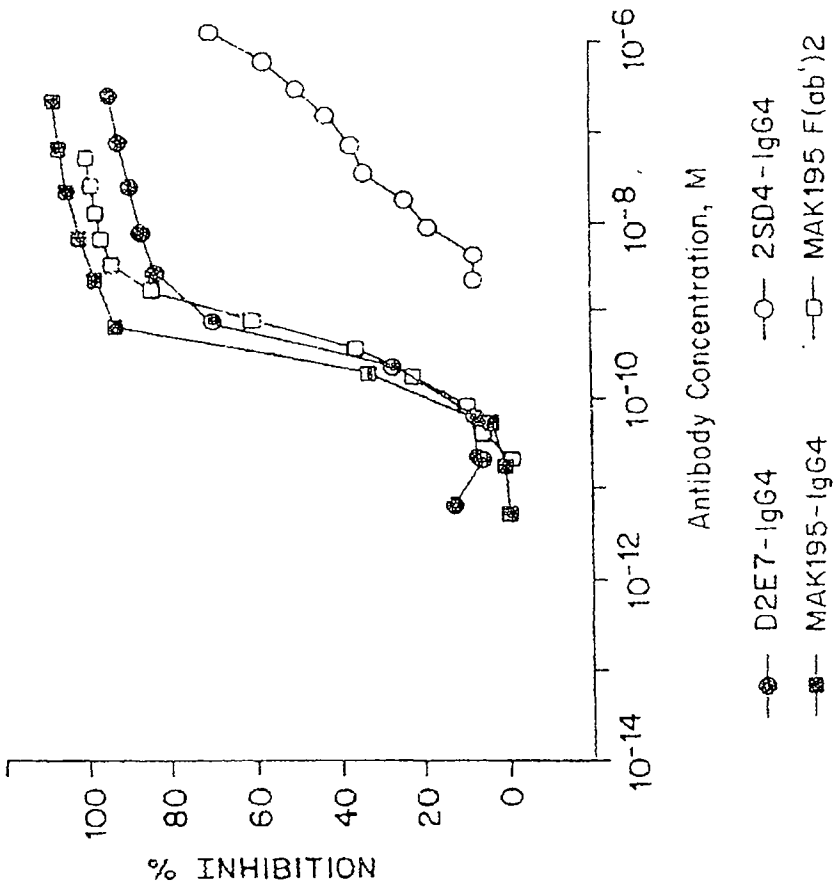

% INHIBITION
100
80
60
40
20
0
10^{-14}
10^{-12}
10^{-10}
10^{-8}
10^{-6}
Antibody Concentration, M
D2E7-IgG4
2SD4-IgG4
MAK195-IgG4
MAK195 F(ab')2

FIG. 5

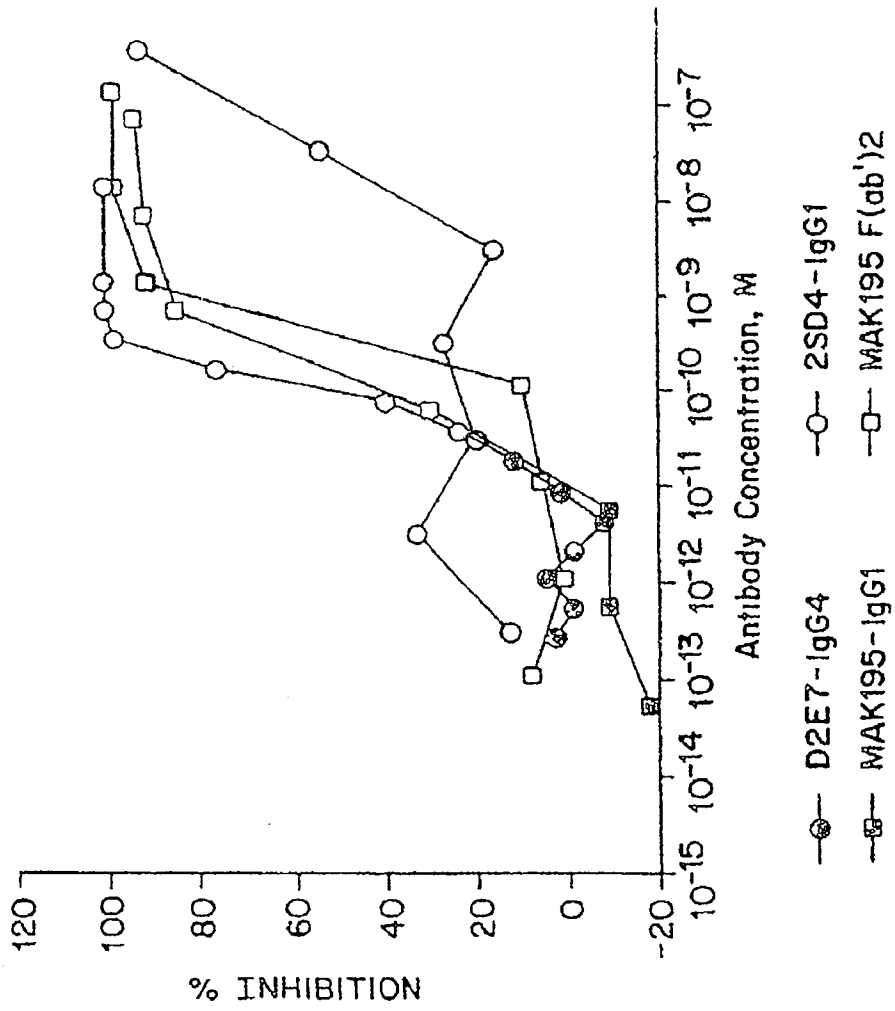
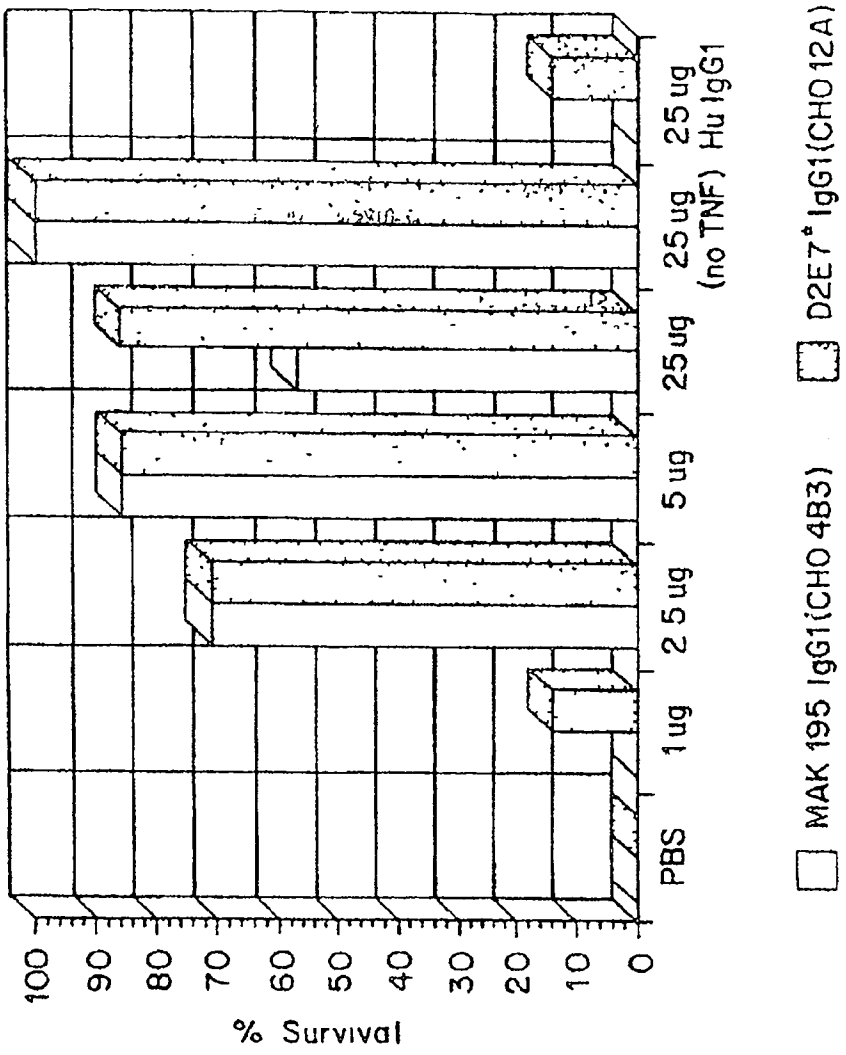

% INHIBITION
120
100
80
60
40
20
0
-20
10^{-15} 10^{-14} 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
Antibody Concentration, M
D2E7-IgG4
2SD4-IgG1
MAK195-IgG1
MAK195 F(ab')2

FIG. 6


100
90
80
70
60
50
40
30
20
10
0
% Survival
PBS
1 ug
2.5 ug
5 ug
25 ug
25 ug (no TNF) Hu IgG1
25 ug
MAK 195 IgG1(CHO 4B3)
D2E7* IgG1(CHO 12A)

D2E7 VL

```
GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA
 D   I   Q   M   T   Q   S   P   S   S   L   S   A   S   V

                                                CDR L1
GGG GAC AGA GTC ACC ATC ACT TGT CGG GCA AGT CAG GGC ATC AGA
 G   D   R   V   T   I   T   C   R   A   S   Q   G   I   R

AAT TAC TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG
 N   Y   L   A   W   Y   Q   Q   K   P   G   K   A   P   K

                                CDR L2
CTC CTG ATC TAT GCT GCA TCC ACT TTG CAA TCA GGG GTC CCA TCT
 L   L   I   Y   A   A   S   T   L   Q   S   G   V   P   S

CGG TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC
 R   F   S   G   S   G   S   G   T   D   F   T   L   T   I

AGC AGC CTA CAG CCT GAA GAT GTT GCA ACT TAT TAC TGT CAA AGG
 S   S   L   Q   P   E   D   V   A   T   Y   Y   C   Q   R

  CDR L3
TAT AAC CGT GCA CCG TAT ACT TTT GGC CAG GGG ACC AAG GTG GAA
 Y   N   R   A   P   Y   T   F   G   Q   G   T   K   V   E

ATC AAA
 I   K
```

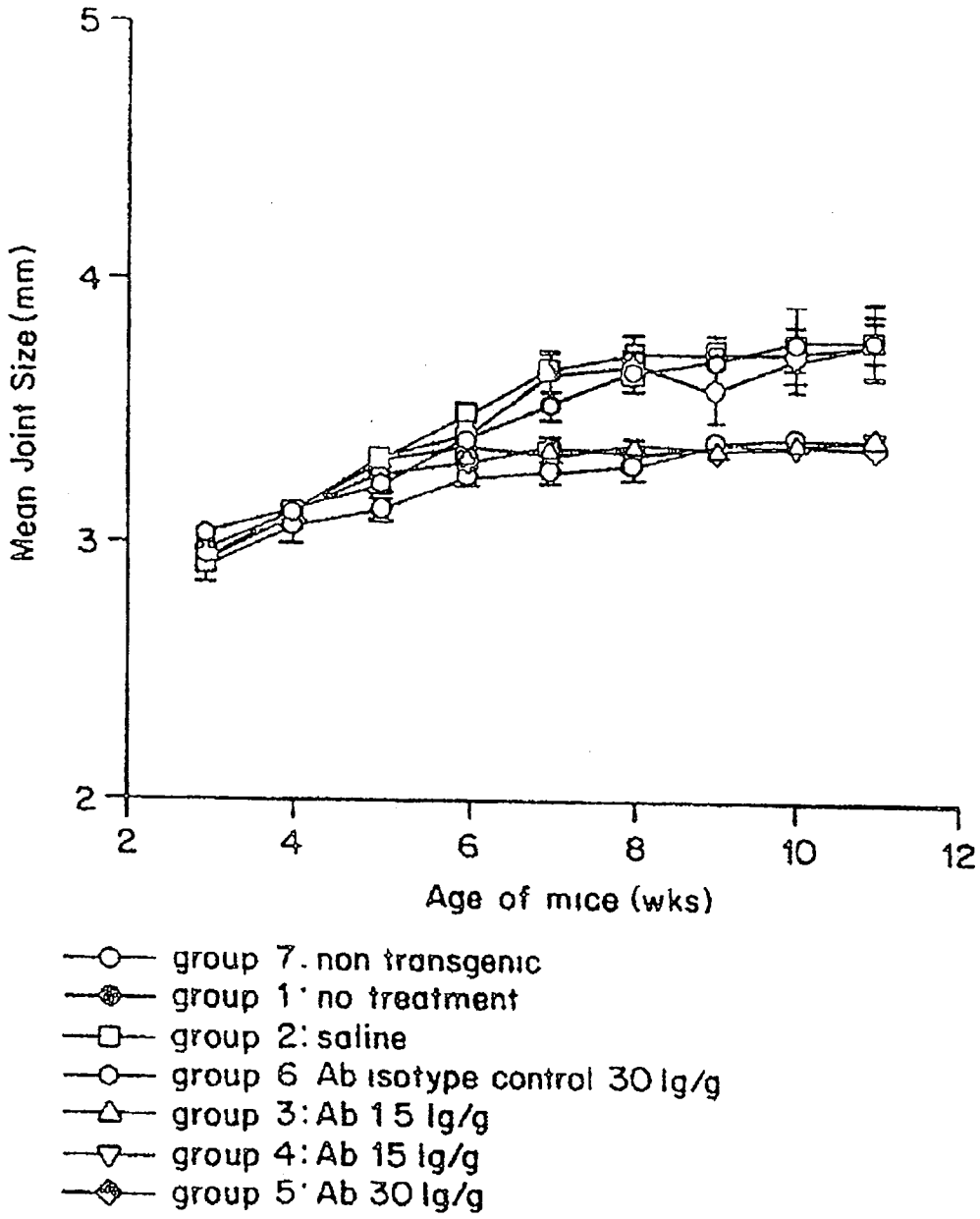
FIGURE 7

D2E7 VH

```
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTA CAG CCC GGC
 E   V   Q   L   V   E   S   G   G   G   L   V   Q   P   G

AGG TCC CTG AGA CTC TCC TGT GCG GCC TCT GGA TTC ACC TTT GAT
 R   S   L   R   L   S   C   A   A   S   G   F   T   F   D

        CDR H1
GAT TAT GCC ATG CAC TGG GTC CGG CAA GCT CCA GGG AAG GGC CTG
 D   Y   A   M   H   W   V   R   Q   A   P   G   K   G   L

                                                CDR H2
GAA TGG GTC TCA GCT ATC ACT TGG AAT AGT GGT CAC ATA GAC TAT
 E   W   V   S   A   I   T   W   N   S   G   H   I   D   Y

GCG GAC TCT GTG GAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC
 A   D   S   V   E   G   R   F   T   I   S   R   D   N   A

AAG AAC TCC CTG TAT CTG CAA ATG AAC AGT CTG AGA GCT GAG GAT
 K   N   S   L   Y   L   Q   M   N   S   L   R   A   E   D

                                                CDR H3
ACG GCC GTA TAT TAC TGT GCG AAA GTC TCG TAC CTT AGC ACC GCG
 T   A   V   Y   Y   C   A   K   V   S   Y   L   S   T   A

TCC TCC CTT GAC TAT TGG GGC CAA GGT ACC CTG GTC ACC GTC TCG
 S   S   L   D   Y   W   G   Q   G   T   L   V   T   V   S

AGT
 S
```

FIGURE 8

FIG. 9


Mean Joint Size (mm)
5
4
3
2
2
4
6
8
10
12
Age of mice (wks)
group 7. non transgenic
group 1: no treatment
group 2: saline
group 6 Ab isotype control 30 lg/g
group 3: Ab 15 lg/g
group 4: Ab 15 lg/g
group 5: Ab 30 lg/g

HUMAN ANTIBODIES THAT BIND HUMAN TNFα

This application is a continuation application of Ser. No. 09/125 098 filed on Mar. 16, 1999, now U.S. Pat. No. 6,258,562 which claims priority from International application Ser. No. PCT/US97/02219 filed Feb. 10, 1997, which claims priority from U.S. provisional application Ser. No. 60/031,476 filed Nov. 25, 1996 and from U.S. application Ser. No. 08/599,226 filed Feb. 9, 1996, now U.S. Pat. No. 6,090,382 the contents of each are expressly incorporated by reference.

BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα) is a cytokine produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see e.g., Old, L. (1985) *Science* 230:630–632). Subsequently, a factor termed cachectin, associated with cachexia, was shown to be the same molecule as TNFα. TNFα has been implicated in mediating shock (see e.g., Beutler, B. and Cerami, A. (1988) *Annu. Rev. Biochem.* 57:505–518; Beutler, B. and Cerami, A. (1989) *Annu. Rev. Immunol.* 7:625–655). Furthermore, TNFα has been implicated in the pathophysiology of a variety of other human diseases and disorders, including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162–169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A., et al Vasilli, P. (1992) *Annu. Rev. Immunol.* 10:411–452; Tracey, K. J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491–503).

Because of the harmful role of human TNFα (hTNFα) in a variety of human disorders, therapeutic strategies have been designed to inhibit or counteract hTNFα activity. In particular, antibodies that bind to, and neutralize, hTNFα have been sought as a means to inhibit hTNFα activity. Some of the earliest of such antibodies were mouse monoclonal antibodies (mAbs), secreted by hybridomas prepared from lymphocytes of mice immunized with hTNFα (see e.g., Hahn T; et al., (1985) *Proc Natl Acad Sci USA* 82: 3814–3818; Liang, C-M., et al. (1986) *Biochem. Biophys. Res. Commun.* 137:847–854; Hirai, M., et al. (1987) *J. Immunol. Methods* 96:57–62; Fendly, B. M., et al. (1987) *Hybridoma* 6:359–370; Moeller, A., et al. (1990) *Cytokine* 2:162–169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 186 833 B1 by Wallach, D.; European Patent Application Publication No. 218 868 A1 by Old et al.; European Patent Publication No. 260 610 B1 by Moeller, A., et al.). While these mouse anti-hTNFα antibodies often displayed high affinity for hTNFα (e.g., $K_d \leqq 10^{-9}$M) and were able to neutralize hTNFα activity, their use in vivo may be limited by problems associated with administration of mouse antibodies to humans, such as short serum half life, an inability to trigger certain human effector functions and elicitation of an unwanted immune response against the mouse antibody in a human (the "human anti-mouse antibody" (HAMA) reaction).

In an attempt to overcome the problems associated with use of fully-murine antibodies in humans, murine anti-hTNFα antibodies have been genetically engineered to be more "human-like." For example, chimeric antibodies, in which the variable regions of the antibody chains are murine-derived and the constant regions of the antibody chains are human-derived, have been prepared (Knight, D. M, et al. (1993) *Mol. Immunol.* 30:1443–1453; PCT Publication No. WO 92/16553 by Daddona, P. E., et al.). Additionally, humanized antibodies, in which the hypervariable domains of the antibody variable regions are murine-derived but the remainder of the variable regions and the antibody constant regions are human-derived, have also been prepared (PCT Publication No. WO 92/11383 by Adair, J. R., et al.). However, because these chimeric and humanized antibodies still retain some murine sequences, they still may elicit an unwanted immune reaction, the human anti-chimeric antibody (HACA) reaction, especially when administered for prolonged periods, e.g., for chronic indications, such as rheumatoid arthritis (see e.g., Elliott, M. J., et al. (1994) *Lancet* 344:1125–1127; Elliot, M. J., et al. (1994) *Lancet* 344:1105–1110).

A preferred hTNFα inhibitory agent to murine mAbs or derivatives thereof (e.g., chimeric or humanized antibodies) would be an entirely human anti-hTNFα antibody, since such an agent should not elicit the HAMA reaction, even if used for prolonged periods. Human monoclonal autoantibodies against hTNFα have been prepared using human hybridoma techniques (Boyle, P., et al. (1993) *Cell. Immunol.* 152:556–568; Boyle, P., et al. (1993) *Cell. Immunol.* 152:569–581; European Patent Application Publication No. 614 984 A2 by Boyle, et al.). However, these hybridoma-derived monoclonal autoantibodies were reported to have an affinity for hTNFα that was too low to calculate by conventional methods, were unable to bind soluble hTNFα and were unable to neutralize hTNFα-induced cytotoxicity (see Boyle, et al.; supra). Moreover, the success of the human hybridoma technique depends upon the natural presence in human peripheral blood of lymphocytes producing autoantibodies specific for hTNFα. Certain studies have detected serum autoantibodies against hTNFα in human subjects (Fomsgaard, A., et al. (1989) *Scand J. Immunol.* 30:219 223; Bendtzen, K., et al. (1990) *Prog. Leukocyte Biol.* 10B:447–452), whereas others have not (Leusch, H-G., et al. (1991) *J. Immunol. Methods* 139:145–147).

Alternative to naturally-occurring human anti-hTNFα antibodies would be a recombinant hTNFα antibody. Recombinant human antibodies that bind hTNFα with relatively low affinity (i.e., $K_d \sim 10^{-7}$M) and a fast off rate (i.e., $K_{off} \sim 10^{-2}$ sec^{-1}) have been described (Griffiths, A. D., et al. (1993) *EMBO J.* 12:725–734). However, because of their relatively fast dissociation kinetics, these antibodies may not be suitable for therapeutic use. Additionally, a recombinant human anti-hTNFα has been described that does not neutralize hTNFα activity, but rather enhances binding of hTNFα to the surface of cells and enhances internalization of hTNFα (Lidbury, A., et al. (1994) *Biotechnol. Ther.* 5:27–45, PCT Publication No. WO 92/03145 by Aston, R. et al.)

Accordingly, human antibodies, such as recombinant human antibodies, that bind soluble hTNFα with high affinity and slow dissociation kinetics and that have the capacity to neutralize hTNFα activity, including hTNFα-induced cytotoxicity (in vitro and in vivo) and hTNFα-induced cell activation, are still needed.

SUMMARY OF THE INVENTION

This invention provides human antibodies, preferably recombinant human antibodies, that specifically bind to human TNFα. The antibodies of the invention are characterized by binding to hTNFα with high affinity and slow dissociation kinetics and by neutralizing hTNFα activity, including hTNFα-induced cytotoxicity (in vitro and in vivo)

and hTNFα-induced cellular activation. Antibodies of the invention are further characterized by binding to hTNFα but not hTNFβ (lymphotoxin) and by having the ability to bind to other primate TNFαs and non-primate TNFαs in addition to human TNFα.

The antibodies of the invention can be full-length (e.g., an IgG1 or IgG4 antibody) or can comprise only an antigen-binding portion (e.g., a Fab, $F(ab')_2$ or scFv fragment). The most preferred recombinant antibody of the invention, termed D2E7, has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 and a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4. Preferably, the D2E7 antibody has a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2.

In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a K_{off} of 1×10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10^{-9} M or less and still more preferably with an IC_{50} of 5×10^{-10} M or less.

In another embodiment, the invention provides a human antibody, or antigen-binding portion thereof, with the following characteristics:

a) dissociates from human TNFα with a K_{off} of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less. Still more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 1×10^{-4} s^{-1} or less.

In yet another embodiment, the invention provides a human antibody, or an antigen-binding portion thereof, with an LCVR having CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with an HCVR having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. More preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6. Still more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with an LCVR comprising the amino acid sequence of SEQ ID NO: 1 and an HCVR comprising the amino acid sequence of SEQ ID NO: 2. In certain embodiments, the antibody has an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. In yet other embodiments, the antibody is a Fab fragment, an $F(ab')_2$ fragment or a single chain Fv fragment.

In still other embodiments, the invention provides antibodies, or antigen-binding portions thereof, with an LCVR having CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or with an HCVR having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, that neutralizes the activity of human TNFα but not human TNFβ (lymphotoxin). In a preferred embodiment, the human antibody, or antigen-binding portion thereof, neutralizes the activity of human TNFα, chimpanzee TNFα and at least one additional primate TNFα selected from the group consisting of baboon TNFα, marmoset TNFα, cynomolgus TNFα and rhesus TNFα. Preferably, the antibody also neutralizes the activity of at least one non-primate TNFα. For example, in one subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of canine TNFα. In another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of pig TNFα. In yet another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of mouse TNFα.

Another aspect of the invention pertains to nucleic acid molecules encoding the antibodies, or antigen-binding portions, of the invention. A preferred nucleic acid of the invention, encoding a D2E7 LCVR, has the nucleotide sequence shown in FIG. 7 and SEQ ID NO 36. Another preferred nucleic acid of the invention, encoding a D2E7 HCVR, has the nucleotide sequence shown in FIG. 8 and SEQ ID NO 37. Recombinant expression vectors carrying the antibody-encoding nucleic acids of the invention, and host cells into which such vectors have been introduced, are also encompassed by the invention, as are methods of making the antibodies of the invention by culturing the host cells of the invention.

Yet another aspect of the invention pertains to methods for inhibiting human TNFα activity using an antibody, or antigen-binding portion thereof, of the invention. In one embodiment, the method comprises contacting human TNFα with the antibody of the invention, or antigen-binding portion thereof, such that human TNFα activity is inhibited. In another embodiment, the method comprises administering an antibody of the invention, or antigen-binding portion thereof, to a human subject suffering from a disorder in which TNFα activity is detrimental such that human TNFα activity in the human subject is inhibited. The disorder can

be, for example, sepsis, an autoimmune disease (e.g., rheumatoid arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome), an infectious disease, a malignancy, transplant rejection or graft-versus-host disease, a pulmonary disorder, a bone disorder, an intestinal disorder or a cardiac disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show the amino acid sequences of the light chain variable region of D2E7 (D2E7 VL; also shown in SEQ ID NO: 1), alanine-scan mutants of D2E7 VL (LD2E7*.A1, LD2E7*.A3, LD2E7*.A4, LD2E7*.A5, LD2E7*.A7 and LD2E7*.A8), the light chain variable region of the D2E7-related antibody 2SD4 (2SD4 VL; also shown in SEQ ID NO: 9) and other D2E7-related light chain variable regions (EP B12, VL10E4, VL100A9, VL100D2, VL10F4, LOE5, VLLOF9, VLL0F10, VLLOG7, VLLOG9, VLLOH1, VLLOH10, VL1B7, VL1C1, VL1C7, VL0.1F4, VL0.1H8, LOE7, LOE7.A and LOE7.T).

FIG. 1A shows the FR1, CDR1, FR2 and CDR2 domains.

FIG. 1B shows the FR3, CDR3 and FR4 domains. The light chain CDR1 ("CDR L1"), CDR2 ("CDR L2") and CDR3 ("CDR L3") domains are boxed.

FIGS. 2A and 2B show the amino acid sequences of the heavy chain variable region of D2E7 (D2E7 VH; also shown in SEQ ID NO: 2), alanine-scan mutants of D2E7 VH (HD2E7*.A1, HD2E7*.A2, HD2E7*.A3, HD2E7*.A4, HD2E7*.A5, HD2E7*.A6, HD2E7*.A7, HD2E7*.A8 and HD2E7*.A9), the heavy chain variable region of the D2E7-related antibody 2SD4 (2SD4 VH; also shown in SEQ ID NO: 10) and other D2E7-related heavy chain variable regions (VH1B11, VH1D8, VH1A11, VH1B12, VH1-D2, VH1E4, VH1F6, VH1G1, 3C-H2, VH1-D2.N and VH1-D2.Y).

FIG. 2A shows the FR1, CDR1, FR2 and CDR2 domains.

FIG. 2B shows the FR3, CDR3 and FR4 domains. The heavy chain CDR1 ("CDR H1"), CDR2 ("CDR H2") and CDR3 ("CDR H3") domains are boxed.

FIG. 3 is a graph depicting the inhibition of TNFα-induced L929 cytotoxicity by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. 4 is a graph depicting the inhibition of rhTNFα binding to hTNFα receptors on U-937 cells by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. 5 is a graph depicting the inhibition of TNFα-induced ELAM-1 expression on HUVEC by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. 6 is a bar graph depicting protection from TNFα-induced lethality in D-galactosamine-sensitized mice by administration of the human anti-hTNFα antibody D2E7 (black bars), as compared to the murine anti-hTNFα antibody MAK 195 (hatched bars).

FIG. 7 shows the nucleotide sequence of the light chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR L1, CDR L2 and CDR L3 regions are underlined.

FIG. 8 shows the nucleotide sequence of the heavy chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR H1, CDR H2 and CDR H3 regions are underlined.

FIG. 9 is a graph depicting the effect of D2E7 antibody treatment on the mean joint size of Tg197 transgenic mice as a polyarthritis model.

DETAILED DESCRIPTION OF THE INVENTION

This invention pertains to isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Various aspects of the invention relate to antibodies and antibody fragments, and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such antibodies and fragments. Methods of using the antibodies of the invention to detect human TNFα or to inhibit human TNFα activity, either in vitro or in vivo, are also encompassed by the invention.

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNFα" (abbreviated herein as hTNFα, or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNFα is described further in, for example, Pennica, D., et al. (1984) *Nature* 312:724–729; Davis, J. M., et al. (1987) *Biochemistry* 26:1322–1326; and Jones, E. Y., et al. (1989) *Nature* 338:225–228. The term human TNFα is intended to include recombinant human TNFα (rhTNFα), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, Minn.).

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, (1989) *Nature* 341:544–546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined,

using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) *Science* 242:423–426; and Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879–5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6444–6448; Poljak, R. J., et al. (1994) *Structure* 2:1121–1123).

Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecules, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S. M., et al. (1995) *Human Antibodies and Hybridomas* 6:93–101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S. M., et al. (1994) *Mol. Immunol.* 31:1047–1058). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further in Section II, below), antibodies isolated from a recombinant, combinatorial human antibody library (described further in Section III, below), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor, L. D., et al. (1992) *Nucl. Acids Res.* 20:6287–6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα). An isolated antibody that specifically binds hTNFα may, however, have cross-reactivity to other antigens, such as TNFα molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody", as used herein (or an "antibody that neutralized hTNFα activity"), is intended to refer to an antibody whose binding to hTNFα results in inhibition of the biological activity of hTNFα. This inhibition of the biological activity of hTNFα can be assessed by measuring one or more indicators of hTNFα biological activity, such as hTNFα-induced cytotoxicity (either in vitro or in vivo), hTNFα-induced cellular activation and hTNFα binding to hTNFα receptors. These indicators of hTNFα biological activity can be assessed by one or more of several standard in vitro or in vivo assays known in the art (see Example 4). Preferably, the ability of an antibody to neutralize hTNFα activity is assessed by inhibition of hTNFα-induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNFα activity, the ability of an antibody to inhibit hTNFα-induced expression of ELAM-1 on HUVEC, as a measure of hTNFα-induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Example 1 and Jönsson, U., et al. (1993) *Ann. Biol. Clin.* 51:19–26; Jönsson, U., et al. (1991) *Biotechniques* 11:620–627; Johnsson, B., et al. (1995) *J. Mol. Recognit.* 8:125–131; and Johnnson, B., et al. (1991) *AnaL Biochem.* 198:268–277.

The term "K_{off}", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_d", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding antibodies or antibody portions (e.g., VH, VL, CDR3) that bind hTNFα, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an

anti-TNFα antibody contains no other sequences encoding other VH regions that bind antigens other than TNFα.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

Various aspects of the invention are described in further detail in the following subsections.

I. Human Antibodies that Bind Human TNFα

This invention provides isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNFα antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 and has VL and VH sequences as shown in FIGS. 1A, 1B and FIGS. 2A, 2B, respectively (the amino acid sequence of the D2E7 VL region is also shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is also shown in SEQ ID NO: 2). The binding properties of D2E7, as compared to the murine anti-hTNFα MAK 195 mAb that exhibits high affinity and slow dissociation kinetics and another human anti-hTNFα antibody related in sequence to D2E7, 2SD4, are summarized below:

Antibody	K_{off} sec^{-1}	k_{on} M^{-1} sec^{-1}	K_d M	Stoichiometry
D2E7 IgG1	8.81×10^{-5}	1.91×10^{5}	6.09×10^{-10}	1.2
2SD4 IgG4	8.4×10^{-3}	4.20×10^{5}	2.00×10^{-8}	0.8
MAK 195 $F(ab')_2$	8.70×10^{-5}	1.90×10^{5}	4.60×10^{-10}	1.4

The D2E7 antibody, and related antibodies, also exhibit a strong capacity to neutralize hTNFα activity, as assessed by several in vitro and in vivo assays (see Example 4). For example, these antibodies neutralize hTNFα-induced cytotoxicity of L929 cells with IC_{50} values in the range of about 10^{-7} M to about 10^{-10} M. D2E7, when expressed as a full-length IgG1 antibody, neutralizes hTNFα-induced cytotoxicity of L929 cells with IC_{50} of about 1.25×10^{-10} M. Moreover, the neutralizing capacity of D2E7 is maintained when the antibody is expressed as a Fab, $F(ab')_2$ or scFv fragment. D2E7 also inhibits TNFα-induced cellular activation, as measured by hTNFα-induced ELAM-1 expression on HUVEC (IC_{50}=about 1.85×10^{-10} M), and binding of hTNFα to hTNFα receptors on U-937 cells (IC_{50}=about 1.56×10^{-10} M). Regarding the latter, D2E7 inhibits the binding of hTNFα to both the p55 and p75 hTNFα receptors. Furthermore, the antibody inhibits hTNFα-induced lethality in vivo in mice (ED_{50}=1–2.5 μg/mouse).

Regarding the binding specificity of D2E7, this antibody binds to human TNFα in various forms, including soluble hTNFα, transmembrane hTNFα and hTNFα bound to cellular receptors. D2E7 does not specifically bind to other cytokines, such as lymphotoxin (TNFβ), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IFNγ and TGFβ. However, D2E7 does exhibit crossreactivity to tumor necrosis factors from other species. For example, the antibody neutralizes the activity of at least five primate TNFαs (chimpanzee, baboon, marmoset, cynomolgus and rhesus) with approximately equivalent IC_{50} values as for neutralization of hTNFα (see Example 4, subsection E). D2E7 also neutralizes the activity of mouse TNFα, although approximately 1000-fold less well than human TNFα (see Example 4, subsection E). D2E7 also binds to canine and porcine TNFα.

In one aspect, the invention pertains to D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a K_{off} of 1×10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10^{-9} M or less and still more preferably with an IC_{50} of 5×10^{-10} M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof. In another preferred embodiment, the antibody also neutralizes TNFα-induced cellular activation, as assessed using a standard in vitro assay for TNFα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC).

Surface plasmon resonance analysis for determining K_d and K_{off} can be performed as described in Example 1. A standard in vitro L929 assay for determining IC_{50} values is described in Example 4, subsection A. A standard in vitro assay for TNFα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC) is described in Example 4, subsection C. Examples of recombinant human

antibodies that meet, or are predicted to meet, the aforementioned kinetic and neutralization criteria include antibodies having the following [VH/VL] pairs, the sequences of which are shown in FIGS. 1A, 1B, 2A and 2B (see also Examples 2, 3 and 4 for kinetic and neutralization analyses): [D2E7 VH/D2E7 VL]; [HD2E7*.A1/D2E7 VL], [HD2E7*.A2/D2E7 VL], [HD2E7*.A3/D2E7 VL], [HD2E7*.A4/D2E7 VL], [HD2E7*.A5/D2E7 VL], [HD2E7*.A6/D2E7 VL], [HD2E7*.A7/D2E7 VL], [HD2E7*.A8/D2E7 VL], [HD2E7*.A9/D2E7 VL], [D2E7 VH/LD2E7*.A1], [D2E7 VH/LD2E7*.A4], [D2E7 VH/LD2E7*.A5], [D2D7 VH/LD2E7*.A7], [D2E7 VH/LD2E7*.A8], [HD2E7*.A9/LD2E7*.A1], [VH1-D2/LOE7], [VH1-D2.N/LOE7.T], [VH1-D2.Y/LOE7.A], [VH1-D2.N/LOE7.A], [VH1-D2/EP B12] and [3C-H2/LOE7].

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to human antibodies that have slow dissociation kinetics for association with hTNFα and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. As demonstrated in Example 3, position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off}. Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNFα. As shown in Example 3, positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNFα and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above).

Accordingly, in another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, with the following characteristics:

a) dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 1×10^{-4} s^{-1} or less.

In yet another embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (i.e., the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (i.e., the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (i.e., the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (i.e., the D2E7 VH CDR1). The framework regions for VL preferably are from the $V_{\kappa}I$ human germline family, more preferably from the A20 human germline Vk gene and most preferably from the D2E7 VL framework sequences shown in FIGS. 1A and 1B. The framework regions for VH preferably are from the VH3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in FIGS. 2A and 2B.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides an isolated human antibody, or an antigen-binding portions thereof, having D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides a recombinant human antibody, or antigen-binding portion thereof, that neutralizes the activity of human TNFα but not human TNFβ. Preferably, antibody, or antigen-binding portion thereof, also neutralizes the activity of chimpanzee TNFα and at least one additional primate TNFα selected from the group consisting of baboon TNFα, marmoset TNFα, cynomolgus TNFα and rhesus TNFα. Preferably, the antibody, or antigen-binding portion thereof, neutralizes the human, chimpanzee and/or additional primate TNFα in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, more preferably 1×10^{-9} M or less, and even more preferably 5×10^{-10} M or less. In one subembodiment, the antibody also neutralizes the activity of canine TNFα, preferably in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less, more preferably 1×10^{-8} M or less and even more preferably 5×10^{-9} M or less. In another subembodiment, the antibody also neutralizes the activity of pig TNFα, preferably with an IC_{50} of 1×10^{-5} M or less, more preferably 1×10^{-6} M or less and even more preferably 5×10^{-7} M or less. In yet another embodiment, the antibody also neutralizes the activity of mouse TNFα, preferably with an IC_{50} of 1×10^{-4} M or less, more preferably 1×10^{-5} M or less and even more preferably 5×10^{-6} M or less.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (e.g., another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNFα antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (e.g., a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, e.g., to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (e.g., disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, Ill.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

II. Expression of Antibodies

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F. M. et al. (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Pat. No. 4,816,397 by Boss et al.

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see e.g., the "Vbase" human germline sequence database; see also Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., et al. (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776–798; and Cox, J. P. L. et al. (1994) "A Directory of Human Germ-line V_κ Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827–836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the $V_\kappa I$ family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31 germline VH and A20 germline VL sequences can be

designed based on the nucleotide sequences disclosed in the references cited supra, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2B7 or D2B7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2B7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Moreover, it should be noted that if the "germline" sequences obtained by PCR amplification encode amino acid differences in the framework regions from the true germline configuration (i.e., differences in the amplified sequence as compared to the true germline sequence, for example as a result of somatic mutation), it may be desireable to change these amino acid differences back to the true germline sequences (i.e., "backmutation" of framework residues to the germline configuration).

Once DNA fragments encoding D2E7 or D2B7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see e.g., Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, e.g., encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see e.g., Bird et al. (1988) *Science* 242:423–426; Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879–5883; McCafferty et al., *Nature* (1990) 348:552–554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH1 segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i e., a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell expression include viral

elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (e.g., the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see e.g., U.S. Pat. No. 5,168,062 by Stinski, U.S. Pat. No. 4,510,245 by Bell et al. and U.S. Pat. No. 4,968,615 by Schaffner et al.

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel et al.). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M. A. and Wood, C. R. (1985) *Immunology Today* 6:12–13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216–4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufman and P. A. Sharp (1982) *Mol. Biol.* 159:601–621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNFα. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNFα by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

In view of the foregoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of the antibodies and antibody portions of the invention. The nucleotide sequence encoding the D2E7 light chain variable region is shown in FIG. 7 and SEQ ID NO: 36. The CDR1 domain of the LCVR encompasses nucleotides 70–102, the CDR2 domain encompasses nucleotides 148–168 and the CDR3 domain encompasses nucleotides 265–291. The nucleotide sequence encoding the D2E7 heavy chain variable region is shown in FIG. 8 and SEQ ID NO: 37. The CDR1 domain of the HCVR encompasses nucleotides 91–105, the CDR2 domain encompasses nucleotides 148–198 and the CDR3 domain encompasses nucleotides 295–330. It will be appreciated by the skilled artisan that nucleotide sequences encoding D2E7-related antibodies, or portions thereof (e.g., a CDR domain, such as a CDR3 domain), can be derived from the nucleotide sequences encoding the D2E7 LCVR and HCVR using the genetic code and standard molecular biology techniques.

In one embodiment, the invention provides an isolated nucleic acid encoding a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 (i.e., the D2E7 VL CDR3), or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9. This nucleic acid can encode only the CDR3 region or, more preferably, encodes an entire antibody light chain variable region (LCVR). For example, the nucleic acid can encode an LCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (i.e., the D2E7 VL CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (i.e., the D2E7 VL CDR1).

In another embodiment, the invention provides an isolated nucleic acid encoding a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4 (i.e., the D2E7 VH CDR3), or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12. This nucleic acid can encode only the CDR3 region or, more preferably, encodes an entire antibody heavy chain variable region (HCVR). For example, the nucleic acid can encode a HCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (i.e., the D2E7 VH CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (i.e., the D2E7 VH CDR1).

In yet another embodiment, the invention provides isolated nucleic acids encoding a D2E7-related CDR3 domain, e.g., comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO 4, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 LCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 36, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 1. The nucleic acid can encode only the LCVR or can also encode an antibody light chain constant region, operatively linked to the LCVR. In one embodiment, this nucleic acid is in a recombinant expression vector.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 HCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 37, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 2. The nucleic acid can encode only the HCVR or can also encode a heavy chain constant region, operatively linked to the HCVR. For example, the nucleic acid can comprise an IgG1 or IgG4 constant region. In one embodiment, this nucleic acid is in a recombinant expression vector.

The invention also provides recombinant expression vectors encoding both an antibody heavy chain and an antibody light chain. For example, in one embodiment, the invention provides a recombinant expression vector encoding:

a) an antibody light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 LCVR); and

b) an antibody heavy chain having a variable region comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 HCVR).

The invention also provides host cells into which one or more of the recombinant expression vectors of the invention have been introduced. Preferably, the host cell is a mammalian host cell, more preferably the host cell is a CHO cell, an NS0 cell or a COS cell.

Still further the invention provides a method of synthesizing a recombinant human antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant human antibody of the invention is synthesized. The method can further comprise isolating the recombinant human antibody from the culture medium.

III. Selection of Recombinant Human Antibodies

Recombinant human antibodies of the invention in addition to the D2E7 or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene SurfZAP™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner et al. U.S. Pat. No. 5,223,409; Kang et al. PCT Publication No. WO 92/18619; Dower et al. PCT Publication No. WO 91/17271; Winter et al. PCT Publication No. WO 92/20791; Markland et al. PCT Publication No. WO 92/15679; Breitling et al. PCT Publication No. WO 93/01288; McCafferty et al. PCT Publication No. WO 92/01047; Garrard et al. PCT Publication No. WO 92/09690; Fuchs et al. (1991) *Bio/Technology* 9:1370–1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81–85; Huse et al. (1989) *Science* 246: 1275–1281; McCafferty et al., *Nature* (1990)348:552–554; Griffiths et al. (1993) *EMBO J.* 12:725–734; Hawkins et al. (1992) *J. Mol Biol* 226:889–896; Clackson et al. (1991) *Nature* 352:624–628; Gram et al. (1992) *PNAS* 89:3576–3580; Garrad et al. (1991) *Bio/Technology* 9:1373–1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133–4137; and Barbas et al. (1991) *PNAS* 88:7978–7982.

In a preferred embodiment, to isolate human antibodies with high affinity and a low off rate constant for hTNFα, a murine anti-hTNFα antibody having high affinity and a low off rate constant for hTNFα. (e.g., MAK 195, the hybridoma for which has deposit number ECACC 87 050801) is first used to select human heavy and light chain sequences having similar binding activity toward hTNFα, using the epitope imprinting, or guided selection, methods described in Hoogenboom et al., PCT Publication No. WO 93/06213. The antibody libraries used in this method are preferably scFv libraries prepared and screened as described in McCafferty et al, PCT Publication No. WO 92/01047, McCafferty et al., *Nature* (1990) 348:552–554; and Griffiths et al., (1993) *EMBO J.* 12:725–734. The scFv antibody libraries preferably are screened using recombinant human TNFα as the antigen.

Once initial human VL and VH segments are selected, "mix and match" experiments, in which different pairs of the initially selected VL and VH segments are screened for hTNFα binding, are performed to select preferred VL/VH pair combinations. Additionally, to further improve the affinity and/or lower the off rate constant for hTNFα binding, the VL and VH segments of the preferred VLNVH pair(s) can be randomly mutated, preferably within the CDR3 region of VH and/or VL, in a process analogous to the in vivo somatic mutation process responsible for affinity maturation of antibodies during a natural immune response.

This in vitro affinity maturation can be accomplished by amplifying VH and VL regions using PCR primers complimentary to the VH CDR3 or VL CDR3, respectively, which primers have been "spiked" with a random mixture of the four nucleotide bases at certain positions such that the resultant PCR products encode VH and VL segments into which random mutations have been introduced into the VH and/or VL CDR3 regions. These randomly mutated VH and VL segments can be rescreened for binding to hTNFα and sequences that exhibit high affinity and a low off rate for hTNFα binding can be selected.

The amino acid sequences of selected antibody heavy and light chains can be compared to germline heavy and light chain amino acid sequences. In cases where certain framework residues of the selected VL and/or VH chains differ from the germline configuration (e.g., as a result of somatic mutation of the immunoglobulin genes used to prepare the phage library), it may be desireable to "backmutate" the altered framework residues of the selected antibodies to the germline configuration (i.e., change the framework amino acid sequences of the selected antibodies so that they are the same as the germline framework amino acid sequences). Such "backmutation" (or "germlining") of framework residues can be accomplished by standard molecular biology methods for introducing specific mutations (e.g., site-directed mutagenesis; PCR-mediated mutagenesis, and the like).

Following screening and isolation of an anti-hTNFα antibody of the invention from a recombinant immunoglobulin display library, nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques. If desired, the nucleic acid can be further manipulated to create other antibody forms of the invention (e.g., linked to nucleic acid encoding additional immunoglobulin domains, such as additional constant regions). To express a recombinant human antibody isolated by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into a mammalian host cells, as described in further detail in Section II above.

IV. Pharmaceutical Compositions and Pharmaceutical Administration

The antibodies and antibody-portions of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody or antibody portion of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody or antibody portion.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies. The preferred mode of administration is parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i e., antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The antibodies and antibody-portions of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, an antibody or antibody portion of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral admin-

istration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating disorders in which TNFα activity is detrimental. For example, an anti-hTNFα antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (e.g., antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see e.g., PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

Nonlimiting examples of therapeutic agents for rheumatoid arthritis with which an antibody, or antibody portion, of the invention can be combined include the following: nonsteroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see e.g., *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see e.g., *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNFR (soluble TNF binding protein; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol.—Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37–42); R973401 (phosphodiesterase Type IV inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39 No. 9 (supplement), S81); Iloprost (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (e.g., Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), 131; *Inflammation Research* (1996) Vol. 45, pp. 103–107); tranexamic acid (inhibitor of plasminogen activation; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see e.g., *Neuro Report* (1996) Vol. 7, pp. 1209–1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitos of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (e.g., SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; interleukin-11 (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see e.g., *Arthritis & Rheumatism* (1996) Vol.39, No. 9 (supplement), S308); interleukin-17 inhibitors (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see e.g., DeLuca et al. (1995) *Rheum. Dis. Clin. North Am.* 21:759–777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); and azaribine.

Nonlimiting examples of therapeutic agents for inflammatory bowel disease with which an antibody, or antibody portion, of the invention can be combined include the following: budenoside; epidermal growth factor; corticosteroids; cyclosporin, sulfasalazine; aminosalicylates; 6-mercaptopurine; azathioprine; metronidazole; lipoxygenase inhibitors; mesalamine; olsalazine; balsalazide; antioxidants; thromboxane inhibitors; IL-1 receptor antagonists; anti-IL-1β monoclonal antibodies; anti-IL-6 monoclonal antibodies; growth factors; elastase inhibitors; pyridinyl-imidazole compounds; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); interleukin-10 (SCH 52000; Schering Plough); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); interleukin-11; glucuronide- or dextran-conjugated prodrugs of prednisolone, dexamethasone or budesonide; ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); slow-release mesalazine; methotrexate; antagonists of Platelet Activating Factor (PAF); ciprofloxacin; and lignocaine.

Nonlimiting examples of therapeutic agents for multiple sclerosis with which an antibody, or antibody portion, of the invention can be combined include the following: corticosteroids; prednisolone; methylprednisolone; azathioprine; cyclophosphamide; cyclosporine; methotrexate; 4-aminopyridine; tizanidine; interferon-β1a (Avonex™; Biogen); interferon-β1b (Betaseron™; Chiron/Berlex); Copolymer 1 (Cop-1; Copaxone™; Teva Pharmaceutical Industries, Inc.); hyperbaric oxygen; intravenous immunoglobulin; clabribine; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IL-10; IL-4; and IL-10 and/or IL-4 agonists (e.g., agonist antibodies).

Nonlimiting examples of therapeutic agents for sepsis with which an antibody, or antibody portion, of the invention can be combined include the following: hypertonic saline solutions; antibiotics; intravenous gamma globulin; continuous hemofiltration; carbapenems (e.g., meropenem); antagonists of cytokines such as TNFα, IL-1β, IL-6 and/or IL-8; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); SK&F 107647 (low molecular peptide; SmithKline Beecham); tetravalent guanylhydrazone CNI-1493 (Picower Institute); Tissue Factor Pathway Inhibitor (TFPI; Chiron); PHP (chemically modified hemoglobin; APEX Bioscience); iron chelators and chelates, including diethylenetriamine pentaacetic acid—iron (III) complex (DTPA iron (III); Molichem Medicines); lisofylline (synthetic small molecule methylxanthine; Cell Therapeutics, Inc.); PGG-Glucan (aqeuous soluble β1,3glucan; Alpha-Beta Technology); apolipoprotein A-1 reconstituted with lipids; chiral hydroxamic acids (synthetic antibacterials that inhibit lipid A biosynthesis); anti-endotoxin antibodies; E5531 (synthetic lipid A antagonist; Eisai America, Inc.); $rBPI_{21}$ (recombinant N-terminal fragment of human Bactericidal/Permeability-Increasing Protein); and Synthetic Anti-Endotoxin Peptides (SAEP; BiosYnth Research Laboratories);

Nonlimiting examples of therapeutic agents for adult respiratory distress syndrome (ARDS) with which an antibody, or antibody portion, of the invention can be combined include the following: anti-IL-8 antibodies; surfactant replacement therapy; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); and 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche).

The use of the antibodies, or antibody portions, of the invention in combination with other therapeutic agents is discussed further in subsection IV.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody or antibody portion may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 0.1–20 mg/kg, more preferably 1–10 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

IV. Uses of the Antibodies of the Invention

Given their ability to bind to hTNFα, the anti-hTNFα antibodies, or portions thereof, of the invention can be used to detect hTNFα (e.g., in a biological sample, such as serum or plasma), using a conventional immunoassay, such as an enzyme linked immunosorbent assays (ELISA), an radioimmunoassay (RIA) or tissue immunohistochemistry. The invention provides a method for detecting hTNFα in a biological sample comprising contacting a biological sample with an antibody, or antibody portion, of the invention and detecting either the antibody (or antibody portion) bound to hTNFα or unbound antibody (or antibody portion), to thereby detect hTNFα in the biological sample. The antibody is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples

of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Alternative to labeling the antibody, hTNFα can be assayed in biological fluids by a competition immunoassay utilizing rhTNFα standards labeled with a detectable substance and an unlabeled anti-hTNFα antibody. In this assay, the biological sample, the labeled rhTNFα standards and the anti-hTNFα antibody are combined and the amount of labeled rhTNFα standard bound to the unlabeled antibody is determined. The amount of hTNFα in the biological sample is inversely proportional to the amount of labeled rhTNFα standard bound to the anti-hTNFα antibody.

A D2E7 antibody of the invention can also be used to detect TNFαs from species other than humans, in particular TNFαs from primates (e.g., chimpanzee, baboon, marmoset, cynomolgus and rhesus), pig and mouse, since D2E7 can bind to each of these TNFαs (discussed further in Example 4, subsection E).

The antibodies and antibody portions of the invention are capable of neutralizing hTNFα activity both in vitro and in vivo (see Example 4). Moreover, at least some of the antibodies of the invention, such as D2E7, can neutralize TNFα activity from other species. Accordingly, the antibodies and antibody portions of the invention can be used to inhibit TNFα activity, e.g., in a cell culture containing hTNFα, in human subjects or in other mammalian subjects having TNFαs with which an antibody of the invention cross-reacts (e.g. chimpanzee, baboon, marmoset, cynomolgus and rhesus, pig or mouse). In one embodiment, the invention provides a method for inhibiting TNFα activity comprising contacting TNFα with an antibody or antibody portion of the invention such that TNFα activity is inhibited. Preferably, the TNFα is human TNFα. For example, in a cell culture containing, or suspected of containing hTNFα, an antibody or antibody portion of the invention can be added to the culture medium to inhibit hTNFα activity in the culture.

In another embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from a disorder in which TNFα activity is detrimental. TNFα has been implicated in the pathophysiology of a wide variety of disorders (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162–169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A.). The invention provides methods for TNFα activity in a subject suffering from such a disorder, which method comprises administering to the subject an antibody or antibody portion of the invention such that TNFα activity in the subject is inhibited. Preferably, the TNFα is human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (e.g., by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time courses of administration).

As used herein, the term "a disorder in which TNFα activity is detrimental" is intended to include diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which TNFα activity is detrimental is a disorder in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (e.g., an increase in the concentration of TNFα in serum, plasma, synovial fluid, etc. of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. There are numerous examples of disorders in which TNFα activity is detrimental. The use of the antibodies and antibody portions of the invention in the treatment of specific disorders is discussed further below:

A. Sepsis

Tumor necrosis factor has an established role in the pathophysiology of sepsis, with biological effects that include hypotension, myocardial suppression, vascular leakage syndrome, organ necrosis, stimulation of the release of toxic secondary mediators and activation of the clotting cascade (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162–169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A.; Tracey, K. J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491–503; Russell, D and Thompson, R. C. (1993) *Curr. Opin. Biotech.* 4:714–721). Accordingly, the human antibodies, and antibody portions, of the invention can be used to treat sepsis in any of its clinical settings, including septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome.

Furthermore, to treat sepsis, an anti-hTNFα antibody, or antibody portion, of the invention can be coadministered with one or more additional therapeutic agents that may further alleviate sepsis, such as an interleukin-1 inhibitor (such as those described in PCT Publication Nos. WO 92/16221 and WO 92/17583), the cytokine interleukin-6 (see e.g., PCT Publication No. WO 93/11793) or an antagonist of platelet activating factor (see e.g., Eropean Patent Application Publication No. EP 374 510). Other combination therapies for the treatment of sepsis are discussed further in subsection III.

Additionally, in a preferred embodiment, an anti-TNFα antibody or antibody portion of the invention is administered to a human subject within a subgroup of sepsis patients having a serum or plasma concentration of IL-6 above 500 pg/ml, and more preferably 1000 pg/ml, at the time of treatment (see PCT Publication No. WO 95/20978 by Daum, L., et al.).

B. Autoimmune Diseases

Tumor necrosis factor has been implicated in playing a role in the pathophysiology of a variety of autoimmune diseases. For example, TNFα has been implicated in activating tissue inflammation and causing joint destruction in rheumatoid arthritis (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162–169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B 1 by Moeller, A.; Tracey and Cerami, supra; Arend, W. P. and Dayer, J-M. (1995) *Arth. Rheum.* 38:151–160; Fava, R. A., et al. (1993) *Clin. Exp. Immunol* 94:261–266). TNFα also has been implicated in promoting the death of islet cells and

in mediating insulin resistance in diabetes (see e.g., Tracey and Cerami, supra; PCT Publication No. WO 94/08609). TNFα also has been implicated in mediating cytotoxicity to oligodendrocytes and induction of inflammatory plaques in multiple sclerosis (see e.g., Tracey and Cerami, supra). Chimeric and humanized murine anti-hTNFα antibodies have undergone clinical testing for treatment of rheumatoid arthritis (see e.g., Elliott, M. J., et al. (1994) *Lancet* 344: 1125-1127; Elliot, M. J., et al. (1994) *Lancet* 344;1105-1110; Rankin, E. C., et al. (1995) *Br. J. Rheumatol.* 34:334–342).

The human antibodies, and antibody portions of the invention can be used to treat autoimmune diseases, in particular those associated with inflammation, including rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome. Typically, the antibody, or antibody portion, is administered systemically, although for certain disorders, local administration of the antibody or antibody portion at a site of inflammation may be beneficial (e.g., local administration in the joints in rheumatoid arthritis or topical application to diabetic ulcers, alone or in combination with a cyclohexaneylidene derivative as described in PCT Publication No. WO 93/19751). An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of autoimmune diseases, as discussed further in subsection III.

C. Infectious Diseases

Tumor necrosis factor has been implicated in mediating biological effects observed in a variety of infectious diseases. For example, TNFα has been implicated in mediating brain inflammation and capillary thrombosis and infarction in malaria. TNFα also has been implicated in mediating brain inflammation, inducing breakdown of the blood-brain barrier, triggering septic shock syndrome and activating venous infarction in meningitis. TNFα also has been implicated in inducing cachexia, stimulating viral proliferation and mediating central nervous system injury in acquired immune deficiency syndrome (AIDS). Accordingly, the antibodies, and antibody portions, of the invention, can be used in the treatment of infectious diseases, including bacterial meningitis (see e.g., European Patent Application Publication No. EP 585 705), cerebral malaria, AIDS and AIDS-related complex (ARC) (see e.g., European Patent Application Publication No. EP 230 574), as well as cytomegalovirus infection secondary to transplantation (see e.g., Fietze, E., et al. (1994) *Transplantation* 58:675–680). The antibodies, and antibody portions, of the invention, also can be used to alleviate symptoms associated with infectious diseases, including fever and myalgias due to infection (such as influenza) and cachexia secondary to infection (e.g., secondary to AIDS or ARC).

D. Transplantation

Tumor necrosis factor has been implicated as a key mediator of allograft rejection and graft versus host disease (GVHD) and in mediating an adverse reaction that has been observed when the rat antibody OKT3, directed against the T cell receptor CD3 complex, is used to inhibit rejection of renal transplants (see e.g., Eason, J. D., et al. (1995) *Transplantation* 59:300–305; Suthanthiran, M. and Strom, T. B. (1994) *New Engl. J. Med.* 331:365–375). Accordingly, the antibodies, and antibody portions, of the invention, can be used to inhibit transplant rejection, including rejections of allografts and xenografts and to inhibit GVHD. Although the antibody or antibody portion may be used alone, more preferably it is used in combination with one or more other agents that inhibit the immune response against the allograft or inhibit GVHD. For example, in one embodiment, an antibody or antibody portion of the invention is used in combination with OKT3 to inhibit OKT3-induced reactions. In another embodiment, an antibody or antibody portion of the invention is used in combination with one or more antibodies directed at other targets involved in regulating immune responses, such as the cell surface molecules CD25 (interleukin-2 receptor-α), CD11a (LFA-1), CD54 (ICAM-1), CD4, CD45, CD28/CTLA4, CD80 (B7-1) and/or CD86 (B7-2). In yet another embodiment, an antibody or antibody portion of the invention is used in combination with one or more general immunosuppressive agents, such as cyclosporin A or FK506.

E. Malignancy

Tumor necrosis factor has been implicated in inducing cachexia, stimulating tumor growth, enhancing metastatic potential and mediating cytotoxicity in malignancies. Accordingly, the antibodies, and antibody portions, of the invention, can be used in the treatment of malignancies, to inhibit tumor growth or metastasis and/or to alleviate cachexia secondary to malignancy. The antibody, or antibody portion, may be administered systemically or locally to the tumor site.

F. Pulmonary Disorders

Tumor necrosis factor has been implicated in the pathophysiology of adult respiratory distress syndrome (ARDS), including stimulating leukocyte-endothelial activation, directing cytotoxicity to pneumocytes and inducing vascular leakage syndrome. Accordingly, the antibodies, and antibody portions, of the invention, can be used to treat various pulmonary disorders, including adult respiratory distress syndrome (see e.g., PCT Publication No. WO 91/04054), shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis and silicosis. The antibody, or antibody portion, may be administered systemically or locally to the lung surface, for example as an aerosol. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of pulmonary disorders, as discussed further in subsection III.

G. Intestinal Disorders

Tumor necrosis factor has been implicated in the pathophysiology of inflammatory bowel disorders (see e.g., Tracy, K. J., et al. (1986) *Science* 234:470–474; Sun, X-M., et al. (1988) *J. Clin. Invest.* 81:1328–1331; MacDonald, T. T., et al (1990) *Clin. Exp. Immunol.* 81:301–305). Chimeric murine anti-hTNFα antibodies have undergone clinical testing for treatment of Crohn's disease (van Dullemen, H. M., et al. (1995) *Gastroenterology* 109:129–135). The human antibodies, and antibody portions, of the invention, also can be used to treat intestinal disorders, such as idiopathic inflammatory bowel disease, which includes two syndromes, Crohn's disease and ulcerative colitis. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of intestinal disorders, as discussed further in subsection III.

H. Cardiac Disorders

The antibodies, and antibody portions, of the invention, also can be used to treat various cardiac disorders, including ischemia of the heart (see e.g., European Patent Application Publication No. EP 453 898) and heart insufficiency (weakness of the heart muscle)(see e.g., PCT Publication No. WO 94/20139).

I. Others

The antibodies, and antibody portions, of the invention, also can be used to treat various other disorders in which TNFα activity is detrimental. Examples of other diseases and disorders in which TNFα activity has been implicated in the pathophysiology, and thus which can be treated using an antibody, or antibody portion, of the invention, include inflammatory bone disorders and bone resorption disease (see e.g., Bertolini, D. R., et al. (1986) *Nature* 319:516–518; Konig, A., et al. (1988) *J. Bone Miner. Res.* 3:621–627; Lemer, U. H. and Ohlin, A. (1993) *J. Bone Miner. Res.* 8:147–155; and Shankar, G. and Stern, P. H. (1993) *Bone* 14:871–876), hepatitis, including alcoholic hepatitis (see e.g., McClain, C. J. and Cohen, D. A. (1989) *Hepatology* 9:349–351; Felver, M. E., et al. (1990) *Alcohol. Clin. Exp. Res.* 14:255–259; and Hansen, J., et al. (1994) *Hepatology* 20:461–474), viral hepatitis (Sheron, N., et al. (1991) *J. Hepatol* 12:241–245; and Hussain, M. J., et al. (1994) *J. Clin. Pathol.* 47:1112-1115), and fulminant hepatitis; coagulation disturbances (see e.g., van der Poll, T., et al. (1990) *N. Engl. J. Med.* 322:1622–1627; and van der Poll, T., et al. (1991) *Prog Clin. Biol. Res.* 367:55–60), burns (see e.g., Giroir, B. P., et al. (1994) *Am. J. Physiol.* 267: H118–124; and Liu, X. S., et al. (1994) *Burns* 20:40–44), reperfusion injury (see e.g., Scales, W. E., et al. (1994) *Am. J. Physiol.* 267:G1122–1127; Serrick, C., et al. (1994) *Transplantation* 58:1158–1162; and Yao, Y. M., et al. (1995) *Resuscitation* 29:157–168), keloid formation (see e.g., McCauley, R. L., et al. (1992) *J. Clin. Immunol.* 12:300–308), scar tissue formation; pyrexia; periodontal disease; obesity and radiation toxicity.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLE 1

Kinetic Analysis of Binding of Human Antibodies to hTNFα

Real-time binding interactions between ligand (biotinylated recombinant human TNFα (rhTNFα) immobilized on a biosensor matrix) and analyte (antibodies in solution) were measured by surface plasmon resonance (SPR) using the BIAcore system (Pharmacia Biosensor, Piscataway, N.J.). The system utilizes the optical properties of SPR to detect alterations in protein concentrations within a dextran biosensor matrix. Proteins are covalently bound to the dextran matrix at known concentrations. Antibodies are injected through the dextran matrix and specific binding between injected antibodies and immobilized ligand results in an increased matrix protein concentration and resultant change in the SPR signal. These changes in SPR signal are recorded as resonance units (RU) and are displayed with respect to time along the y-axis of a sensorgram.

To facilitate immobilization of biotinylated rhTNFα on the biosensor matrix, streptavidin is covalently linked via free amine groups to the dextran matrix by first activating carboxyl groups on the matrix with 100 mM N-hydroxysuccinimide (NHS) and 400 mM N-ethyl-N'-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC). Next, streptavidin is injected across the activated matrix. Thirty-five microliters of streptavidin (25 μg/ml), diluted in sodium acetate, pH 4.5, is injected across the activated biosensor and free amines on the protein are bound directly to the activated carboxyl groups. Unreacted matrix EDC-esters are deactivated by an injection of 1 M ethanolamine. Streptavidin-coupled biosensor chips also are commercially available (Pharmacia BR-1000-16, Pharmacia Biosensor, Piscataway, N.J.).

Biotinylated rhTNFα was prepared by first dissolving 5.0 mg of biotin (D-biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester; Boehringer Mannheim Cat. No. 1008 960) in 500 μl dimethylsulfoxide to make a 10 mg/ml solution. Ten microliters of biotin was added per ml of rhTNFα (at 2.65 mg/ml) for a 2:1 molar ratio of biotin to rhTNFα. The reaction was mixed gently and incubated for two hours at room temperature in the dark. A PD-10 column, Sephadex G-25M (Pharmacia Catalog No. 17-0851-01) was equilibrated with 25 ml of cold PBS and loaded with 2 ml of rhTNFα-biotin per column. The column was eluted with 10×1 ml cold PBS. Fractions were collected and read at OD280 (1.0 OD=1.25 mg/ml). The appropriate fractions were pooled and stored at −80° C. until use. Biotinylated rhTNFα also is commercially available (R & D Systems Catalog No. FTA00, Minneapolis, Minn.).

Biotinylated rhTNFα to be immobilized on the matrix via streptavidin was diluted in PBS running buffer (Gibco Cat. No. 14190-144, Gibco BRL, Grand Island, N.Y.) supplemented with 0.05% (BIAcore) surfactant P20 (Pharmacia BR-1000–54, Pharmacia Biosensor, Piscataway, N.J.). To determine the capacity of rhTNFα-specific antibodies to bind immobilized rhTNFα, a binding assay was conducted as follows. Aliquots of biotinylated rhTNFα (25 nM; 10 μl aliquots) were injected through the streptavidin-coupled dextran matrix at a flow rate of 5 μl/min. Before injection of the protein and immediately afterward, PBS buffer alone flowed through each flow cell. The net difference in signal between baseline and approximately 30 sec. after completion of biotinylated rhTNFα injection was taken to represent the binding value (approximately 500 RU). Direct rhTNFα-specific antibody binding to immobilized biotinylated rhTNFα was measured. Antibodies (20 μg/ml) were diluted in PBS running buffer and 25 μl aliquots were injected through the immobilized protein matrices at a flow rate of 5 μl/min. Prior to injection of antibody, and immediately afterwards, PBS buffer alone flowed through each flow cell. The net difference in baseline signal after completion of antibody injection was taken to represent the binding value of the particular sample. Biosensor matrices were regenerated using 100 mM HCl before injection of the next sample. To determine the off rate (K_{off}), on rate (K_{on}), association rate (K_a) and dissociation rate (K_d) constants, BIAcore kinetic evaluation software (version 2.1) was used.

Representative results of D2E7 (IgG4 full-length antibody) binding to biotinylated rhTNFα, as compared to the mouse mAb MAK 195 ($F(ab')_2$ fragment), are shown below in Table 1.

TABLE 1

Binding of D2E7 IgG4 or MAK 195 to Biotinylated rhTNFα

Antibody	[Ab], nM	rhTNFα, bound, RUs	Ab, bound, RUs	rhTNFα/ Ab	K_{off} sec^{-1}, (Avg)
D2E7	267	373	1215	1.14	8.45×10^{-5}
	133	420	1569	1.30	5.42×10^{-5}
	67	434	1633	1.31	4.75×10^{-5}
	33	450	1532	1.19	4.46×10^{-5}
	17	460	1296	0.98	3.47×10^{-5}
	8	486	936	0.67	2.63×10^{-5}

TABLE 1-continued

Binding of D2E7 IgG4 or MAK 195 to Biotinylated rhTNFα

Antibody	[Ab], nM	rhTNFα, bound, RUs	Ab, bound, RUs	rhTNFα/ Ab	K_{off}, sec^{-1}, (Avg)
	4	489	536	0.38	2.17×10^{-5}
	2	470	244	0.18	3.68×10^{-5}
					(4.38×10^{-5})
MAK 195	400	375	881	1.20	5.38×10^{-5}
	200	400	1080	1.38	4.54×10^{-5}
	100	419	1141	1.39	3.54×10^{-5}
	50	427	1106	1.32	3.67×10^{-5}
	25	446	957	1.09	4.41×10^{-5}
	13	464	708	0.78	3.66×10^{-5}
	6	474	433	0.47	7.37×10^{-5}
	3	451	231	0.26	6.95×10^{-5}
					(4.94×10^{-5})

In a second series of experiments, the molecular kinetic interactions between an IgG1 full-length form of D2E7 and biotinylated rhTNF was quantitatively analyzed using BIAcore technology, as described above, and kinetic rate constants were derived, summarized below in Tables 2, 3 and 4.

TABLE 2

Apparent dissociation rate constants of the interaction between D2E7 and biotinylated rhTNF

Experiment	K_d (s^{-1})
1	9.58×10^{-5}
2	9.26×10^{-5}
3	7.60×10^{-5}
Average	$8.81 \pm 1.06 \times 10^{-5}$

TABLE 3

Apparent association rate constants of the interaction between D2E7 and biotinylated rhTNF

Experiment	K_a (M^{-1}, s^{-1})
1	1.33×10^5
2	1.05×10^5
3	3.36×10^5
Average	$1.91 \pm 1.26 \times 10^5$

TABLE 4

Apparent kinetic reate and affinity constants of D2E7 and biotinylated rhTNF

Experiment	K_a (M^{-1}, s^{-1})	K_d (s^{-1})	K_d (M)
1	1.33×10^5	9.58×10^{-5}	7.20×10^{-10}
2	1.05×10^5	9.26×10^{-5}	8.82×10^{-10}
3	3.36×10^5	7.60×10^{-5}	2.26×10^{-10}
Average	$1.91 \pm 1.26 \times 10^5$	$8.81 \pm 1.06 \times 10^{-5}$	$6.09 \pm 3.42 \times 10^{-10}$

Dissociation rate constants were calculated by analyzing the dissociation and association regions of the sensorgrams by BIA analysis software. Conventional chemical reaction kinetics were assumed for the interaction between D2E7 and biotinylated rhTNF molecule: a zero order dissociation and first order association kinetics. For the sake of analysis, interaction only between one arm of the bivalent D2E7 antibody and one unit of the trimeric biotinylated rhTNF was considered in choosing molecular models for the analysis of the kinetic data. Three independent experiments were performed and the results were analyzed separately. The average apparent dissociation rate constant (k_d) of the interaction between D2E7 and biotinylated rhTNF was $8.81 \pm 1.06 \times 10^{-5}$ s^{-1}, and the average apparent association rate constant, k_a was $1.91 \pm 1.26 \times 10^5$ M^{-1} s^{-1}. The apparent intrinsic dissociation constant (K_d) was then calculated by the formula: $K_d = k_d/k_a$. Thus, the mean K_d of D2E7 antibody for rhTNF derived from kinetic parameters was $6.09 \pm 3.42 \times 10^{-10}$ M. Minor differences in the kinetic values for the IgG1 form of D2E7 (presented in Tables 2, 3 and 4) and the IgG4 form of D2E7 (presented in Table 1 and in Examples 2 and 3) are not thought to be true differences resulting from the presence of either an IgG1 or an IgG4 constant regions but rather are thought to be attributable to more accurate antibody concentration measurements used for the IgG1 kinetic analysis. Accoringly, the kinetic values for the IgG1 form of D2E7 presented herein are thought to be the most accurate kinetic parameters for the D2E7 antibody.

EXAMPLE 2

Alanine Scanning Mutagenesis of D2E7 CDR3 Domains

A series of single alanine mutations were introduced by standard methods along the CDR3 domain of the D2E7 VL and the D2E7 VH regions. The light chain mutations are illustrated in FIG. 1B (LD2E7*.A1, LD2E7*.A3, LD2E7*.A4, LD2E7*.A5, LD2E7*.A7 and LD2E7*.A8, having an alanine mutation at position 1, 3, 4, 5, 7 or 8, respectively, of the D2E7 VL CDR3 domain). The heavy chain mutations are illustrated in FIG. 2B (HD2E7*.A1, HD2E7*.A2, HD2E7*.A3, HD2E7*.A4, HD2E7*.A5, HD2E7*.A6, HD2E7*.A7, HD2E7*.A8 and HD2E7*.A9, having an alanine mutation at position 2, 3, 4, 5, 6, 8, 9, 10 or 11, respectively, of the D2E7 VH CDR3 domain). The kinetics of rhTNFα interaction with an antibody composed of wild-type D2E7 VL and VH was compared to that of antibodies composed of 1) a wild-type D2E7 VL paired with an alanine-substituted D2E7 VH; 2) a wild-type D2E7 VH paired with an alanine-substituted D2E7 VL; or 3) an alanine-substituted D2E7 VL paired with an alanine-substituted D2E7 VH. All antibodies were tested as full-length, IgG4 molecules.

Kinetics of interaction of antibodies with rhTNFα was determined by surface plasmon resonance as described in Example 1. The K_{off} rates for the different VH/VL pairs are summarized below in Table 5:

Table 5

Binding of D2E7 Alanine-Scan Mutants to Biotinylated rhTNFα

VH	VL	K_{off}(sec^{-1})
D2E7 VH	D2E7 VL	9.65×10^{-5}
HD2E7*.A1	D2E7 VL	1.4×10^{-4}
HD2E7*.A2	D2E7 VL	4.6×10^{-4}
HD2E7*.A3	D2E7 VL	8.15×10^{-4}
HD2E7*.A4	D2E7 VL	1.8×10^{-4}
HD2E7*.A5	D2E7 VL	2.35×10^{-4}
HD2E7*.A6	D2E7 VL	2.9×10^{-4}
HD2E7*.A7	D2E7 VL	1.0×10^{-4}
HD2E7*.A8	D2E7 VL	3.1×10^{-4}
HD2E7*.A9	D2E7 VL	8.1×10^{-4}
D2E7 VH	LD2E7*.A1	6.6×10^{-5}
D2E7 VH	LD2E7*.A3	NOT DETECTABLE

Table 5-continued

Binding of D2E7 Alanine-Scan Mutants to Biotinylated rhTNFα

VH	VL	$K_{off}(sec^{-1})$
D2E7 VH	LD2E7*.A4	1.75×10^{-4}
D2E7 VH	LD2E7*.A5	1.8×10^{-4}
D2E7 VH	LD2E7*.A7	1.4×10^{-4}
D2E7 VH	LD2E7*.A8	3.65×10^{-4}
HD2E7*.A9	LD2E7*.A1	1.05×10^{-4}

These results demonstrate that the majority of positions of the CDR3 domains of the D2E7 VL region and VH region are amenable to substitution with a single alanine residue. Substitution of a single alanine at position 1, 4, 5, or 7 of the D2E7 VL CDR3 domain or at position 2, 5, 6, 8, 9 or 10 of the D2E7 VH CDR3 domain does not significantly affect the off rate of hTNFα binding as compared to the wild-type parental D2E7 antibody. Substitution of alanine at position 8 of the D2E7 VL CDR3 or at position 3 of the D2E7 VH CDR3 gives a 4-fold faster K_{off} and an alanine substitution at position 4 or 11 of D2E7 VH CDR3 gives an 8-fold faster K_{off}, indicating that these positions are more critical for binding to hTNFα. However, a single alanine substitution at position 1, 4, 5, 7 or 8 of the D2E7 VL CDR3 domain or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 of the D2E7 VH CDR3 domain still results in an anti-hTNFα antibody having a K_{off} of 1×10^{-3} sec^{-1} or less.

EXAMPLE 3

Binding Analysis of D2E7-Related Antibodies

A series of antibodies related in sequence to D2E7 were analyzed for their binding to rhTNFα, as compared to D2E7, by surface plasmon resonance as described in Example 1. The amino acid sequences of the VL regions tested are shown in FIGS. 1A and 1B. The amino acid sequences of the VH regions tested are shown in FIGS. 2A and 2B. The K_{off} rates for various VH/VL pairs (in the indicated format, either as a full-length IgG1 or IgG4 antibody or as a scFv) are summarized below in Table 6:

TABLE 6

Binding of D2E7-Related Antibodies to Biotinylated rhTNFα

VH	VL	Format	$K_{off}(sec^{-1})$
D2E7 VH	D2E7 VL	IgG1/IgG4	9.65×10^{-5}
VH1-D2	LOE7	IgG1/IgG4	7.7×10^{-5}
VH1-D2	LOE7	scFv	4.6×10^{-4}
VH1-D2.N	LOE7.T	IgG4	2.1×10^{-5}
VH1-D2.Y	LOE7.A	IgG4	2.7×10^{-5}
VH1-D2.N	LOE7.A	IgG4	3.2×10^{-5}
VH1-D2	EP B12	scFv	8.0×10^{-4}
VH1-D2	2SD4 VL	scFv	1.94×10^{-3}
3C-H2	LOE7	scFv	1.5×10^{-3}
2SD4 VH	LOE7	scFv	6.07×10^{-3}
2SD4 VH	2SD4 VL	scFv	1.37×10^{-2}
VH1A11	2SD4 VL	scFv	1.34×10^{-2}
VH1B12	2SD4 VL	scFv	1.01×10^{-2}
VH1B11	2SD4 VL	scFv	9.8×10^{-3}
VH1E4	2SD4 VL	scFv	1.59×10^{-2}
VH1F6	2SD4 VL	scFv	2.29×10^{-2}
VH1D8	2SD4 VL	scFv	9.5×10^{-3}
VH1G1	2SD4 VL	scFv	2.14×10^{-2}
2SD4 VH	EP B12	scFv	6.7×10^{-3}
2SD4 VH	VL10E4	scFv	9.6×10^{-3}
2SD4 VH	VL100A9	scFv	1.33×10^{-2}
2SD4 VH	VL100D2	scFv	1.41×10^{-2}

TABLE 6-continued

Binding of D2E7-Related Antibodies to Biotinylated rhTNFα

VH	VL	Format	$K_{off}(sec^{-1})$
2SD4 VH	VL10F4	scFv	1.11×10^{-2}
2SD4 VH	VLLOE5	scFv	1.16×10^{-2}
2SD4 VH	VLLOF9	scFv	6.09×10^{-3}
2SD4 VH	VLLOF10	scFv	1.34×10^{-2}
2SD4 VH	VLLOG7	scFv	1.56×10^{-2}
2SD4 VH	VLLOG9	scFv	1.46×10^{-2}
2SD4 VH	VLLOH1	scFv	1.17×10^{-2}
2SD4 VH	VLLOH10	scFv	1.12×10^{-2}
2SD4 VH	VL1B7	scFv	1.3×10^{-2}
2SD4 VH	VL1C1	scFv	1.36×10^{-2}
2SD4 VH	VL1C7	scFv	2.0×10^{-2}
2SD4 VH	VL0.1F4	scFv	1.76×10^{-2}
2SD4 VH	VL0.1H8	scFv	1.14×10^{-2}

The slow off rates (i.e., $K_{off} \leqq 1\times10^{-4}$ sec^{-1}) for full-length antibodies (i.e., IgG format) having a VL selected from D2E7, LOE7, LOE7.T and LOE7.A, which have either a threonine or an alanine at position 9, indicate that position 9 of the D2E7 VL CDR3 can be occupied by either of these two residues without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Furthermore, the slow off rates (i.e., $K_{off} < 1\times10^{-4}$ sec^{-1}) for antibodies having a VH selected from D2E7, VH 1-D2.N and VH1-D2.Y, which have either a tyrosine or an asparagine at position 12, indicate that position 12 of the D2E7 VH CDR3 can be occupied by either of these two residues without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4).

The results shown in Table 6 demonstrate that, in scFv format, antibodies containing the 2SD4 VL or VH CDR3 region exhibit a faster K_{off} (i.e., $K_{off} \geqq 1\times10^{-3}$ sec^{-1}) as compared to antibodies containing the D2E7 VL or VH CDR3 region. Within the VL CDR3, 2SD4 differs from D2E7 at positions 2, 5 and 9. As discussed above, however, position 9 may be occupied by Ala (as in 2SD4) or Thr (as in D2E7) without substantially affecting the K_{off}. Thus, by comparison of 2SD4 and D2E7, positions 2 and 5 of the D2E7 VL CDR3, both arginines, can be identified as being critical for the association of the antibody with hTNFα. These residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Regarding the importance of position 2, replacement of Arg (in LOE7, which has the same VL CDR3 as D2E7) with Lys (in EP B12) accelerates the off rate by a factor of two. Regarding the importance of position 5, replacement of Arg (in D2E7) with Ala (in LD2E7*.A5), as described in Example 2, also accelerates the off rate two-fold. Furthermore, without either Arg at positions 2 and 5 (in 2SD4), the off rate is five-fold faster. However, it should be noted that although position 5 is important for improved binding to hTNFα, a change at this position can be negated by changes at other positions, as seen in VLLOE4, VLLOH1 or VL0.1H8.

Within the VH CDR3, 2SD4 differs from D2E7 at positions 1, 7 and 12. As discussed above, however, position 12 may be occupied by Asn (as in 2SD4) or Tyr (as in D2E7) without substantially affecting the K_{off}. Thus, by comparison of 2SD4 and D2E7, positions 1 and 7 of the D2E7 VH CDR3 can be identified as being critical for binding to hTNFα. As

discussed above, these residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Both positions are important for binding to hTNFα since when the 3C-H2 VH CDR3 (which has a valine to alanine change at position 1 with respect to the D2E7 VH CDR3) is used, the scFv has a 3-fold faster off rate than when the D2E7 VH CDR3 is used but this off rate is still four times slower than when the 2SD4 VH CDR3 is used (which has changes at both positions 1 and 7 with respect to the D2E7 VH CDR3).

EXAMPLE 4

Functional Activity of D2E7

To examine the functional activity of D2E7, the antibody was used in several assays that measure the ability of the antibody to inhibit hTNFα activity, either in vitro or in vivo.

A. Neutralization of TNFα-Induced Cytotoxicity in L929 Cells

Human recombinant TNFα (rhTNFα) causes cell cytotoxicity to murine L929 cells after an incubation period of 18–24 hours. Human anti-hTNFα antibodies were evaluated in L929 assays by coincubation of antibodies with rhTNFα and the cells as follows. A 96-well microtiter plate containing 100 μl of anti-hTNFα Abs was serially diluted ½ down the plate in duplicates using RPMI medium containing 10% fetal bovine serum (FBS). Fifty microliters of rhTNFα was added for a final concentration of 500 pg/ml in each sample well. The plates were then incubated for 30 minutes at room temperature. Next, 50 μl of TNFα-sensitive L929 mouse fibroblasts cells were added for a final concentration of 5×10^4 cells per well, including 1 μg/ml Actinomycin-D. Controls included medium plus cells and rhTNFα plus cells. These controls, and a TNFα standard curve, ranging from 2 ng/ml to 8.2 pg/ml, were used to determine the quality of the assay and provide a window of neutralization. The plates were then incubated overnight (18–24 hours) at 370° C. in 5% CO_2.

One hundred microliters of medium was removed from each well and 50 μl of 5 mg/ml 3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT; commercially available from Sigma Chemical Co., St. Louis, Mo.) in PBS was added. The plates were then incubated for 4 hours at 37° C. Fifty microliters of 20% sodium dodecyl sulfate (SDS) was then added to each well and the plates were incubated overnight at 37° C. The optical density at 570/630 nm was measured, curves were plotted for each sample and IC_{50}s were determined by standard methods.

Representative results for human antibodies having various VL and VH pairs, as compared to the murine MAK 195 mAb, are shown in FIG. 3 and in Table 7 below.

TABLE 7

Neutralization of TNFα-Induced L929 Cytotoxicity

VH	VL	Structure	IC_{50}, M
D2E7	D2E7	scFv	1.1×10^{-10}
D2E7	D2E7	IgG4	4.7×10^{-11}
2SD4	2SD4	scFv/IgG1/IgG4	3.0×10^{-7}
2SD4	LOE7	scFv	4.3×10^{-8}
VH1-D2	2SD4	scFv	1.0×10^{-8}
VH1-D2	LOE7	scFv/IgG1/IgG4	3.4×10^{-10}
VH1.D2.Y	LOE7.T	IgG4	8.1×10^{-11}
VH1-D2.N	LOE7.T	IgG4	1.3×10^{-10}

TABLE 7-continued

Neutralization of TNFα-Induced L929 Cytotoxicity

VH	VL	Structure	IC_{50}, M
VH1-D2.Y	LOE7.A	IgG4	2.8×10^{-11}
VH1-D2.N	LOE7.A	IgG4	6.2×10^{-11}
MAK 195	MAK 195	scFv	1.9×10^{-8}
MAK 195	MAK 195	$F(ab')_2$	6.2×10^{-11}

The results in FIG. 3 and Table 7 demonstrate that the D2E7 human anti-hTNFα antibody, and various D2E7-related antibodies, neutralize TNFα-induced L929 cytotoxicity with a capacity approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to neutralize TNFα-induced L929 cytotoxicity was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 8:

TABLE 8

Neutralization of TNFα-Induced L929 Cytotoxicity by D2E7 IgG1

Experiment	IC_{50} [M]
1	1.26×10^{-10}
2	1.33×10^{-10}
3	1.15×10^{-10}
Average	$1.25 \pm 0.01 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, neutralizes TNFα-induced L929 cytotoxicity with an average IC_{50} [M] of $1.25\pm0.01\times10^{-10}$.

B. Inhibition of TNFα Binding to TNFα Receptors on U-937 Cells

The ability of human anti-hTNFα antibodies to inhibit the binding of hTNFα to hTNFα receptors on the surface of cells was examined using the U-937 cell line (ATCC No. CRL 1593), a human histiocytic cell line that expresses hTNFα receptors. U-937 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone A-1111, Hyclone Laboratories, Logan, UT), L-glutamine (4 nM), HEPES buffer solution (10 mM), penicillin (100 μg/ml) and streptomycin (100 μg/ml). To examine the activity of full-length IgG antibodies, U-937 cells were preincubated with PBS supplemented with 1 mg/ml of human IgG (Sigma I-4506, Sigma Chemical Co., St. Louis, Mo.) for 45 minutes on ice and then cells were washed three times with binding buffer. For the receptor binding assay, U-937 cells (5×10^6 cells/well) were incubated in a binding buffer (PBS supplemented with 0.2% bovine serum albumin) in 96-well microtiter plates (Costar 3799, Costar Corp., Cambridge, Mass.) together with ^{125}I-labeled rhTNFα (3×10^{-10} M; 25 μCi/ml; obtained from NEN Research Products, Wilmington, Del.), with or without anti-hTNFα antibodies. in a total volume of 0.2 ml. The plates were incubated on ice for 1.5 hours. Then, 75 μl of each sample was transferred to 1.0 ml test tubes (Sarstedt 72.700, Sarstedt Corp., Princeton, N.J.) containing dibutylphthalate (Sigma D-2270, Sigma Chemical Co., St. Louis, Mo.) and dinonylphthalate (ICN 210733, ICN, Irvine, Calif.). The test tubes contained a 300 μl mixture of dibutylphthalate and dinonylphthalate, 2:1 volume ratio, respectively. Free (i.e., unbound) ^{125}I-labeled rhTNFα was removed by microcen-

trifugation for five minutes. Then, each test tube end containing a cell pellet was cut with the aid of a microtube scissor (Bel-Art 210180001, Bel-Art Products, Pequannock, N.J.). The cell pellet contains ^{125}I-labeled rhTNFα, bound to the p60 or p80 TNFα receptor, whereas the aqueous phase above the oil mixture contains excess free ^{125}I-labeled rhTNFα. All cell pellets were collected in a counting tube (Falcon 2052, Becton Dickinson Labware, Lincoln Park, N.J.) and counted in a scintillation counter.

Representative results are shown in FIG. **4**. The IC_{50} value for D2E7 inhibition of hTNFα binding to hTNFα receptors on U-937 cells is approximately 3×10^{-10} M in these experiments. These results demonstrate that the D2E7 human anti-hTNFα antibody inhibits rhTNFα binding to hTNFα receptors on U-937 cells at concentrations approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit rhTNFα binding to hTNFα receptors on U-937 cells was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 9:

TABLE 9

Inhibition of TNF Receptor Binding on U-937 Cells by D2E7 IgG1

Experiment	IC_{50} [M]
1	1.70×10^{-10}
2	1.49×10^{-10}
3	1.50×10^{-10}
Average	$1.56 \pm 0.12 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNF receptor binding on U-937 cells with an average IC_{50} [M] of $1.56\pm0.12\times10^{-10}$.

To investigate the inhibitory potency of D2E7 in the binding of ^{125}I-rhTNF binding to individual p55 and p75 receptors, a solid phase radioimmunoassay was performed. To measure the IC_{50} values of D2E7 for separate TNF receptors, varying concentrations of the antibody were incubated with 3×10^{-10} concentration of ^{125}I-rhTNF. The mixture was then tested on separate plates containing either the p55 or the p75 TNF receptors in a dose dependent manner. The results are summarized below in Table 10:

TABLE 10

Inhibition of TNF Receptor Binding to p55 and p75 TNFR by D2E7 IgG1

	IC_{50} [M]	
Reagent	p55 TNFR	p 75TNFR
D2E7	1.47×10^{-9}	1.26×10^{-9}
rhTNF	2.31×10^{-9}	2.70×10^{-9}

Inhibition of ^{125}I-rhTNF binding to the p55 and p75 TNF receptors on U937 cells by D2E7 followed a simple sigmoidal curve, indicating similar IC_{50} values for each receptor. In the solid phase radioimmunoassay (RIA) experiments with recombinant TNF receptors, IC_{50} values for inhibition of ^{125}I-rhTNF binding to the p55 and the p75 receptors by D2E7 were calculated as 1.47×10^{-9} and 1.26×10^{-9} M, respectively. The decrease in IC_{50} values in the solid phase was probably due to higher density of receptors in the RIA format, as unlabeled rhTNF also inhibited with similar IC_{50} values. The IC_{50} values for inhibition of ^{125}I-rhTNF binding to the p55 and the p75 receptors by unlabeled rhTNF were 2.31×10^{-9} and 2.70×10^{-9} M, respectively

C. Inhibition of ELAM-1 Expression on HUVEC

Human umbilical vein endothelial cells (HUVEC) can be induced to express endothelial cell leukocyte adhesion molecule 1 (ELAM-1) on their cell-surface by treatment with rhTNFα, which can be detected by reacting rhTNFα-treated HUVEC with an mouse anti-human ELAM-1 antibody. The ability of human anti-hTNFα antibodies to inhibit this TNFα-induced expression of ELAM-1 on HUVEC was examined as follows: HUVEC (ATCC No. CRL 1730) were plated in 96-well plates (5×10^4 cells/well) and incubated overnight at 37° C. The following day, serial dilutions of human anti-hTNFα antibody (1:10) were prepared in a microtiter plate, starting with 20–100 μg/ml of antibody. A stock solution of rhTNFα was prepared at 4.5 ng/ml, aliquots of rhTNFα were added to each antibody-containing well and the contents were mixed well. Controls included medium alone, medium plus anti-hTNFα antibody and medium plus rhTNFα. The HUVEC plates were removed from their overnight incubation at 37° C. and the medium gently aspirated from each well. Two hundred microliters of the antibody-rhTNFα mixture were transferred to each well of the HUVEC plates. The HUVEC plates were then further incubated at 37° C. for 4 hours. Next, a murine anti-ELAM-1 antibody stock was diluted 1:1000 in RPMI. The medium in each well of the HUVEC plate was gently aspirated, 50 μl/well of the anti-ELAM-1 antibody solution was added and the HUVEC plates were incubated 60 minutes at room temperature. An ^{125}I-labeled anti-mouse Ig antibody solution was prepared in RPMI (approximately 50,000 cpm in 50 μl). The medium in each well of the HUVEC plates was gently aspirated, the wells were washed twice with RPMI and 50 μl of the ^{125}I-labeled anti-mouse Ig solution was added to each well. The plates were incubated for one hour at room temperature and then each well was washed three times with RPMI. One hundred eighty microliters of 5% SDS was added to each well to lyse the cells. The cell lysate from each well was then transferred to a tube and counted in a scintillation counter.

Representative results are shown in FIG. **5**. The IC_{50} value for D2E7 inhibition of hTNFα-induced expression of ELAM-1 on HUVEC is approximately 6×10^{-11} M in these experiments. These results demonstrate that the D2E7 human anti-hTNFα antibody inhibits the hTNFα-induced expression of ELAM-1 on HUVEC at concentrations approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit hTNFα-induced expression of ELAM-1 on HUVEC was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 11:

TABLE 11

Inhibition of TNFα-Induced ELAM-1 Expression by D2E7 IgG1 Receptor

Experiment	IC_{50} [M]
1	1.95×10^{-10}
2	1.69×10^{-10}
3	1.90×10^{-10}
Average	$1.85 \pm 0.14 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNFα-induced ELAM-1 expression on HUVEC with an average IC_{50} [M] of $1.85\pm0.14\times10^{-10}$.

The neutralization potency of D2E7 IgG1 was also examined for the rhTNF induced expression of two other adhesion molecules, ICAM-1 and VCAM-1. Since the rhTNF titration curve for ICAM-1 expression at 16 hours was very similar to the curve of ELAM-1 expression, the same concentration of rhTNF was used in the antibody neutralization experiments. The HUVEC were incubated with rhTNF in the presence of varying concentrations of D2E7 in a 37° C. CO_2 incubator for 16 hours, and the ICAM-1 expression was measured by mouse anti-ICAM-1 antibody followed by ^{125}I-labeled sheep anti-mouse antibody. Two independent experiments were performed and the IC_{50} values were calculated. An unrelated human IgG1 antibody did not inhibit the ICAM-1 expression.

The experimental procedure to test inhibition of VCAM-1 expression was the same as the procedure for ELAM-1 expression, except anti-VCAM-1 MAb was used instead of anti-ELAM-1 MAb. Three independent experiments were performed and the IC_{50} values were calculated. An unrelated human IgG1 antibody did not inhibit VCAM-1 expression.

The results are summarized below in Table 12:

TABLE 12

Inhibition of ICAM-1 and VCAM-1 Expression by D2E7 IgG1

ICAM-1 Inhibition		IC_{50} [M]	
Experiment	IC_{50} [M]	Experiment	IC_{50} [M]
1	1.84×10^{-10}	1	1.03×10^{-10}
2	2.49×10^{-10}	2	9.26×10^{-11}
		3	1.06×10^{-10}
Average	$2.17 \pm 0.46 \times 10^{-10}$	Average	$1.01 \pm 0.01 \times 10^{-10}$

These experiments demonstrate that treatment of primary human umbilical vein endothelial cells with rhTNF led to optimum expression of adhesion molecules: ELAM-1 and VCAM-1 at four hours, and the maximum up-regulated expression of ICAM-1 at 16 hours. D2E7 was able to inhibit the expression of the three adhesion molecules in a dose dependent manner. The IC_{50} values for the inhibition of ELAM-1, ICAM-1 and VCAM-1 were 1.85×10^{-10}, 2.17×10^{-10} and 1.01×10^{-10} M, respectively. These values are very similar, indicating similar requirements for the dose of rhTNF activation signal to induce ELAM-1, ICAM-1 and VCAM-1 expression. Interestingly, D2E7 was similarly effective in the longer inhibition assay of the the ICAM-1 expression. The ICAM-1 inhibition assay required 16 hours of co-incubation of rhTNF and D2E7 with HUVEC as opposed to 4 hours required for the ELAM-1 and the VCAM-1 inhibition assays. Since D2E7 has a slow off-rate for rhTNF, it is conceivable that during the 16 hour co-incubation period there was no significant competition by the TNF receptors on the HUVEC.

D. In vivo Neutralization of hTNFα

Three different in vivo systems were used to demonstrate that D2E7 is effective at inhibiting hTNFα activity in vivo.

1. Inhibition of TNF-Induced Lethality in D-Galactosamine-Sensitized Mice

Injection of recombinant human TNFα (rhTNFα) to D-galactosamine sensitized mice causes lethality within a 24 hour time period. TNFα neutralizing agents have been shown to prevent lethality in this model. To examine the ability of human anti-hTNFα antibodies to neutralize hTNFα in vivo in this model, C57B1/6 mice were injected with varying concentrations of D2E7-IgG1, or a control protein, in PBS intraperitoneally (i.p.). Mice were challenged 30 minutes later with 1 μg of rhTNFα and 20 mg of D-galactosamine in PBS i.p., and observed 24 hours later. These amount of rhTNFα and D-galactosamine were previously determined to achieve 80–90% lethality in these mice.

Representative results, depicted as a bar graph of % survival versus antibody concentration, are shown in FIG. 6. The black bars represent D2E7, whereas the hatched bars represent MAK 195. Injection of 2.5–25 μg of D2E7 antibody per mouse protected the animals from TNFα-induced lethality. The ED_{50} value is approximately 1–2.5 μg/mouse. The positive control antibody, MAK 195, was similar in its protective ability. Injection of D2E7 in the absence of rhTNFα did not have any detrimental effect on the mice. Injection of a non-specific human IgG1 antibody did not offer any protection from TNFα-induced lethality.

In a second experiment, forty-nine mice were divided into 7 equal groups. Each group received varying doses of D2E7 thirty minutes prior to receiving an LD_{80} dose of rhTNF/D-galactosamine mixture (1.0 μg rhTNF and 20 mg D-galactosamine per mouse). Control group 7 received normal human IgG1 kappa antibody at 25 μg/mouse dose. The mice were examined 24 hours later. Survival for each group is summarized below in Table 13.

TABLE 13

24 Hour Survival After Treatment with D2E7

Group	Survival (alive/total)	Survival (%)
1 (no antibody)	0/7	0
2 (1 μg)	1/7	14
3 (2.6 μg)	5/7	71
4 (5.2 μg)	6/7	86
5 (26 μg)	6/7	86
6 (26 μg; no rhTNF)	7/7	100
7 (25 μg Hu IgG1)	1/7	14

II. Inhibition of TNF-Induced Rabbit Pyrexia

The efficacy of D2E7 in inhibiting rhTNF-induced pyrexia response in rabbits was examined. Groups of three NZW female rabbits weighing approximately 2.5 kg each were injected intravenously with D2E7, rhTNF, and immune complexes of D2E7 and rhTNF. Rectal temperatures were measured by thermistor probes on a Kaye thermal recorder every minute for approximately 4 hours. Recombinant human TNF in saline, injected at 5 μg/kg, elicted a rise in temperature greater than 0.4° C. at approximately 45 minutes after injection. The antibody preparation by itself, in saline at a dose of 138 μg/kg, did not elicit a rise in temperature in the rabbits up to 140 minutes after administration. In all further experiments, D2E7 or control reagents (human IgG1 or a saline vehicle) were injected i.v. into rabbits followed 15 minutes later by an injection of rhTNF in saline at 5 μg/kg i.v. Representative results of several experiments are summarized below in Table 14:

TABLE 14

Inhibition of rhTNF-induced Pyrexia with D2E7 in Rabbits

	Temp. rise*, ° C.			Molar Ratio	Peak Temp.
D2E7 dose (μg/kg)	rhTNF	rhTNF + D2E7	% Inhib.**	D2E7: rhTNF	minutes post rhTNF
14	0.53	0.25	53	1	60
24	0.43	0.13	70	1.6	40
48	0.53	0.03	94	3.3	50
137	0.53	0.00	100	9.5	60
792	0.80	0.00	100	55	60

*= Peak temperature

**= % inhibition = (1-{temperature rise with rhTNF & D2E7/temperature rise with rhTNF alone}) × 100.

Intravenous pretreatment with D2E7 at a dose of 14 μg/kg partially inhibited the pyrogenic response, compared to rabbits pre-treated with saline alone. D2E7 administered at 137 μg/kg totally suppressed the pyrogenic response of rhTNF in the same experiment. In a second experiment, D2E7 administered at 24 μg/kg also partially suppressed the pyrogenic response, compared to rabbits pretreated with saline alone. The molar ratio of D2E7 to rhTNF was ½:1 in this experiment. In a third experiment, D2E7 injected i.v. at 48 μg/kg (molar ratio D2E7:rhTNF=3.3:1) totally suppressed the pyrogenic response, compared to rabbits pretreated with the control human IgG1 in saline at 30 μg/kg. In the final experiment, rabbits pretreated with D2E7 (792 μg/kg) at a very high molar ratio to rhTNF (55:1) did not develop any rise in temperature at any time up to 4 hours of observation. Treatment of rabbits with immune complexes generated from a mixture of D2E7 and rhTNF incubated at 37° C. for 1 hour at a molar ratio of 55:1, without subsequent rhTNF administration, also did not elicit any rise in temperature in the same experiment.

III. Prevention of Polyarthritis in Tg197 Transgenic Mice

The effect of D2E7 on disease development was investigated in a transgenic murine model of arthritis. Transgenic mice (Tg197) have been generated that express human wild type TNF (modified in the 3' region beyond the coding sequences) and these mice develop chronic polyarthritis with 100% incidence at 4–7 weeks of age (see *EMBO J.* (1991) 10:4025–4031 for further description of the Tg197 model of polyarthritis).

Transgenic animals were identified by PCR at 3 days of age Litters of transgenic mice were divided into six groups. Transgenic mice were verified by slot-blot hybridization analysis at 15 days of age. The treatment protocols for the six groups were as follows: Group 1=no treatment; Group 2=saline (vehicle); Group 3=D2E7 at 1.5 μg/g; Group 4=D2E7 at 15 μg/g; Group 5=D2E7 at 30 μg/g; and Group 6=IgG1 isotype control at 30 μg/g. A litter with non transgenic mice was also included in the study to serve as a control (Group 7—nontransgenic; no treatment). Each group received three i.p. injections per week of the indicated treatments. Injections continued for 10 weeks. Each week, macroscopic changes in joint morphology were recorded for each animal. At 10 weeks, all mice were sacrificed and mouse tissue was collected in formalin. Microscopic examination of the tissue was performed.

Animal weight in grams was taken for each mouse at the start of each week. At the same time measurements of joint size (in mm) were also taken, as a measurement of disease severity. Joint size was established as an average of three measurements on the hind right ankle using a micrometer device. Arthritic scores were recorded weekly as follows: 0=No arthritis, (normal appearence and flexion); +=mild arthritis (joint distortion); ++=moderate arthritis (swelling, joint deformation) and +++=heavy arthritis (ankylosis detected on flexion and severely impaired movement). Histopathological scoring based on haematoxylin/eosin staining of joint sections was based as follows; 0=No detectable disease; 1=proliferation of the synovial membrane; 2=heavy synovial thickening 3=cartilage destruction and bone erosion.

The effect of D2E7 treatment on the mean joint size of the Tg 197 transgenic arthritic mice is shown in the graph of FIG. 9. The histopathological and arthritic scores of the Tg197 transgenic mice, at 11 weeks of age, are summarized below in Table 15:

TABLE 15

Effect of D2E7 on Histopathology and Arthritic Score in Tg197 Mice

Group	Treatment	Histopathological Score	Arthritic Score
1	none	3 (7/7)	+++ (7/7)
2	saline	3 (8/8)	+++ (8/8)
6	IgG1 control	3 (9/9)	+++ (7/9)
3	D2E7 at 1.5 μg/g	0 (6/8)	0 (8/8)
4	D2E7 at 15 μg/g	0 (7/8)	0 (8/8)
5	D2E7 at 30 μg/g	0 (8/8)	0 (8/8)

This experiment demonstrated that the D2E7 antibody has a definite beneficial effect on transgenic mice expressing the wild-type human TNF (Tg197) with no arthritis evident after the study period.

E. D2E7 Neutralization of TNFαs from Other Species

The binding specificity of D2E7 was examined by measuring its ability to neutralize tumor necrosis factors from various primate species and from mouse, using an L929 cytotoxicity assay (as described in Example 4, subsection A, above). The results are summarized in Table 16 below:

TABLE 16

Ability of D2E7 to Neutralize TNF from Different Species in the L929 Assay

TNFα*	Source	IC_{50} for D2E7 Neutralization (M)**
Human	Recombinant	7.8×10^{-11}
Chimpanzee	LPS-stimulated PBMC	5.5×10^{-11}
baboon	Recombinant	6.0×10^{-11}
marmoset	LPS-stimulated PBMC	4.0×10^{-10}

TABLE 16-continued

Ability of D2E7 to Neutralize TNF from Different Species in the L929 Assay

TNFα*	Source	IC_{50} for D2E7 Neutralization (M)**
cynomolgus	LPS-stimulated PBMC	8.0×10^{-11}
rhesus	LPS-stimulated PBMC	3.0×10^{-11}
canine	LPS-stimulated WBC	2.2×10^{-10}
porcine	Recombinant	1.0×10^{-7}
murine	Recombinant	$>1.0 \times 10^{-7}$

The results in Table 16 demonstrate that D2E7 can neutralize the activity of five primate TNFαs approximately equivalently to human TNFα and, moreover, can neutralize the activity of canine TNFα (about ten-fold less well than human TNFα) and porcine and mouse TNFα (about~1000-fold less well than human TNFα). Moreover, the binding of D2E7 to solution phase rhTNFα was not inhibited by other cytokines, such as lymphotoxin (TNFβ), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IFNγ and TGFβ, indicating that D2E7 is very specific for its ligand TNFα.

F. Lack of Cytokine Release by Human Whole Blood Incubated with D2E7

In this example, the ability of D2E7 to induce, by itself, normal human blood cells to secrete cytokines or shed cell surface molecules was examined. D2E7 was incubated with diluted whole blood from three different normal donors at varying concentrations for 24 hours. An LPS positive control was run at the same time, at a concentration previously determined to stimulate immunocompetent blood cells to secrete cytokines. The supernatants were harvested and tested in a panel of ten soluble cytokine, receptor and adhesion molecule ELISA kits: IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNFα, soluble TNF receptor I, soluble TNF receptor II, soluble ICAM-1 and soluble E-selectin. No significant amounts of cytokines or shed cell surface molecules were measured as a result of D2E7 antibody co-incubation, at concentrations up to 343 μg/ml. Control cultures without the addition of the antibody also did not yield any measurable amounts of cytokines, whereas the LPS co-culture control yielded elevated values in the high picogram to low nanogram range. These results indicate that D2E7 did not induce whole blood cells to secrete cytokines or shed cell surface proteins above normal levels in ex vivo cultures.

Forming part of the present disclosure is the appended Sequence Listing, the contents of which are summarized in the table below:

SEQ ID NO:	ANTIBODY CHAIN	REGION	SEQUENCE TYPE
1	D2E7	VL	amino acid
2	D2E7	VH	amino acid
3	D2E7	VL CDR3	amino acid
4	D2E7	VH CDR3	amino acid
5	D2E7	VL CDR2	amino acid
6	D2E7	VH CDR2	amino acid
7	D2E7	VL CDR1	amino acid
8	D2E7	VH CDR1	amino acid
9	2SD4	VL	amino acid
10	2SD4	VH	amino acid
11	2SD4	VL CDR3	amino acid
12	EP B12	VL CDR3	amino acid
13	VL10E4	VL CDR3	amino acid
14	VL100A9	VL CDR3	amino acid
15	VLL100D2	VL CDR3	amino acid
16	VLL0F4	VL CDR3	amino acid
17	LOE5	VL CDR3	amino acid
18	VLLOG7	VL CDR3	amino acid
19	VLLOG9	VL CDR3	amino acid
20	VLLOH1	VL CDR3	amino acid
21	VLLOH10	VL CDR3	amino acid
22	VL1B7	VL CDR3	amino acid
23	VL1C1	VL CDR3	amino acid
24	VL0.1F4	VL CDR3	amino acid
25	VL0.1H8	VL CDR3	amino acid
26	LOE7.A	VL CDR3	amino acid
27	2SD4	VH CDR3	amino acid
28	VH1B11	VH CDR3	amino acid
29	VH1D8	VH CDR3	amino acid
30	VE1A11	VH CDR3	amino acid
31	VH1B12	VH CDR3	amino acid
32	VH1E4	VH CDR3	amino acid
33	VH1F6	VH CDR3	amino acid
34	3C-H2	VH CDR3	amino acid
35	VH1-D2.N	VH CDR3	amino acid
36	D2E7	VL	nucleic acid
37	D2E7	VH	nucleic acid

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation. many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 37

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-continued

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Arg Tyr Asn Arg Ala Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr
            20                  25                  30

Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val
    50                  55                  60

Glu Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 9

-continued

(D) OTHER INFORMATION: /note= "Xaa is Thr or Ala"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gln Arg Tyr Asn Arg Ala Pro Tyr Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 12
(D) OTHER INFORMATION: /note= "Xaa is Tyr or Asn"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ala Ala Ser Thr Leu Gln Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val Gl
1 5 10 15

Gly

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

-continued

Arg Ala Ser Gln Gly Ile Arg Asn Tyr Leu Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Asp Tyr Ala Met His
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Ile Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Lys Tyr Asn Ser Ala Pro Tyr
85 90 95

Ala Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asp Asp Tyr
20 25 30

Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Asp Trp Val
35 40 45

Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val

-continued

```
    50                  55                  60

Glu Gly Arg Phe Ala Val Ser Arg Asp Asn Ala Lys Asn Ala Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Thr Lys Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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Gln Lys Tyr Asn Ser Ala Pro Tyr Ala
1               5
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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Gln Lys Tyr Asn Arg Ala Pro Tyr Ala
1               5
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Gln Lys Tyr Gln Arg Ala Pro Tyr Thr
1               5
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

-continued

Gln Lys Tyr Ser Ser Ala Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Gln Lys Tyr Asn Ser Ala Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Gln Lys Tyr Asn Arg Ala Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Gln Lys Tyr Asn Ser Ala Pro Tyr Tyr
1 5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Gln Lys Tyr Asn Ser Ala Pro Tyr Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Gln Lys Tyr Thr Ser Ala Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gln Lys Tyr Asn Arg Ala Pro Tyr Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Gln Lys Tyr Asn Ser Ala Ala Tyr Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Gln Gln Tyr Asn Ser Ala Pro Asp Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Gln Lys Tyr Asn Ser Asp Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 24:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Gln Lys Tyr Ile Ser Ala Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Gln Lys Tyr Asn Arg Pro Pro Tyr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gln Arg Tyr Asn Arg Ala Pro Tyr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

-continued

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Ala Ser Tyr Leu Ser Thr Ser Phe Ser Leu Asp Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu His Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Ala Ser Phe Leu Ser Thr Ser Ser Ser Leu Glu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Ala Ser Tyr Leu Ser Thr Ala Ser Ser Leu Glu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 321 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GACATCCAGA TGACCCAGTC TCCATCCTCC CTGTCTGCAT CTGTAGGGGA CAGAGTCACC 60

ATCACTTGTC GGGCAAGTCA GGGCATCAGA AATTACTTAG CCTGGTATCA GCAAAAACCA 120

GGGAAAGCCC CTAAGCTCCT GATCTATGCT GCATCCACTT TGCAATCAGG GGTCCCATCT 180

CGGTTCAGTG GCAGTGGATC TGGGACAGAT TTCACTCTCA CCATCAGCAG CCTACAGCCT 240

GAAGATGTTG CAACTTATTA CTGTCAAAGG TATAACCGTG CACCGTATAC TTTTGGCCAG 300

GGGACCAAGG TGGAAATCAA A 321

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 363 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GAGGTGCAGC TGGTGGAGTC TGGGGGAGGC TTGGTACAGC CCGGCAGGTC CCTGAGACTC 60

TCCTGTGCGG CCTCTGGATT CACCTTTGAT GATTATGCCA TGCACTGGGT CCGGCAAGCT 120

CCAGGGAAGG GCCTGGAATG GGTCTCAGCT ATCACTTGGA ATAGTGGTCA CATAGACTAT 180

GCGGACTCTG TGGAGGGCCG ATTCACCATC TCCAGAGACA ACGCCAAGAA CTCCCTGTAT 240

CTGCAAATGA ACAGTCTGAG AGCTGAGGAT ACGGCCGTAT ATTACTGTGC GAAAGTCTCG 300

TACCTTAGCA CCGCGTCCTC CCTTGACTAT TGGGGCCAAG GTACCCTGGT CACCGTCTCG 360

AGT 363

The invention claimed is:

1. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate, such that the rheumatoid arthritis is treated, wherein the antibody is an isolated human antibody, or an antigen-binding portion thereof, tat dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less.

2. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of 5×10^{-4} s^{-1} or less.

3. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of 1×10^{-4} s^{-1} or less.

4. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less.

5. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotaxicity in a standard in vitro L929 assay wit an IC_{50} of 1×10^{-9} M or less.

6. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-10} M or less.

7. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, is a recombinant antibody, or antigen-binding portion thereof.

8. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate such that the rheumatoid arthritis is treated, wherein the antibody is D2E7.

9. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate, such that the rheumatoid arthritis is treated, wherein the antibody is an isolated human antibody, or antigen-binding portion thereof, with the following characteristics:

a) dissociates from human INFα with a K_{off} rate constant 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1,4, 5, 7 or 8 or by one to five conservative amino avid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

10. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate, such that the rheumatoid arthritis is treated, wherein the antibody is an isolated human antibody, or an antigen binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,223,394 B2
APPLICATION NO. : 09/801185
DATED : May 29, 2007
INVENTOR(S) : Jochen G. Salfeld et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On Title Page Item (63) Please insert

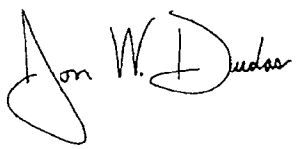
Continuation of application No. 09/125,098, filed on Mar. 16, 1999, now Pat. No. 6,258,562, which is a U.S. National Stage of PCT/US97/02219, filed on Feb. 10, 1997, which claims the benefit of 60/031,476, filed on Nov. 25, 1996, and is a continuation of 08/599,226, filed on Feb. 9, 1996, now Pat. No. 6,090,382.

Instead of

Continuation of application No. 09/125,098, filed on Mar. 16, 1999, now Pat. No. 6,258,562.

Signed and Sealed this

Eighteenth Day of November, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT B

US007541031B2

(12) **United States Patent**
Salfeld et al.

(10) **Patent No.: US 7,541,031 B2**
(45) **Date of Patent: *Jun. 2, 2009**

(54) **METHODS FOR TREATING RHEUMATOID ARTHRITIS USING HUMAN ANTIBODIES THAT BIND HUMAN TNFα**

(75) Inventors: **Jochen G. Salfeld**, North Grafton, MA (US); **Deborah J. Allen**, London (GB); **Zehra Kaymakcalan**, Westborough, MA (US); **Boris Labkovsky**, Marlborough, MA (US); **John A. Mankovich**, Andover, MA (US); **Brian T. McGuinness**, Cambridge (GB); **Andrew J. Roberts**, Cambridge (GB); **Paul Sakorafas**, Newton Highlands, MA (US); **Hendricus R. J. M. Hoogenboom**, Hasselt (BE); **David Schoenhaut**, Clifton, NJ (US); **Tristan J. Vaughan**, Cambridge (GB); **Michael White**, Framingham, MA (US); **Alison J. Wilton**, Cambridge (GB)

(73) Assignee: **Abbott Biotechnology Ltd.** (BM)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **11/787,901**

(22) Filed: **Apr. 17, 2007**

(65) **Prior Publication Data**

US 2007/0249813 A1 Oct. 25, 2007

Related U.S. Application Data

(63) Continuation of application No. 09/801,185, filed on Mar. 7, 2001, now Pat. No. 7,223,394, which is a continuation of application No. 09/125,098, filed as application No. PCT/US97/02219 on Feb. 10, 1997, now Pat. No. 6,258,562, which is a continuation-in-part of application No. 08/599,226, filed on Feb. 9, 1996, now Pat. No. 6,090,382.

(60) Provisional application No. 60/031,476, filed on Nov. 25, 1996.

(51) **Int. Cl.**
A61K 39/395 (2006.01)
A61K 31/495 (2006.01)

(52) **U.S. Cl.** **424/142.1**; 424/145.1; 424/158.1; 424/810; 514/249

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner—David A Saunders
(74) *Attorney, Agent, or Firm* McCarter & English, LLP; Elizabeth A. Hanley, Esq.; Cristin Howley Cowles

(57) **ABSTRACT**

Human antibodies, preferably recombinant human antibodies, that specifically bind to human tumor necrosis factor α (hTNFα) are disclosed. These antibodies have high affinity for hTNFα (e.g., K_d=10^{-8} M or less), a slow off rate for hTNFα dissociation (e.g., K_{off}=10^{-3} sec^{-1} or less) and neutralize hTNFα activity in vitro and in vivo. An antibody of the invention can be a full-length antibody or an antigen-binding portion thereof. The antibodies, or antibody portions, of the invention are useful for detecting hTNFα and for inhibiting hTNFα activity, e.g., in a human subject suffering from a disorder in which hTNFα activity is detrimental. Nucleic acids, vectors and host cells for expressing the recombinant human antibodies of the invention, and methods of synthesizing the recombinant human antibodies, are also encompassed by the invention.

11 Claims, 11 Drawing Sheets

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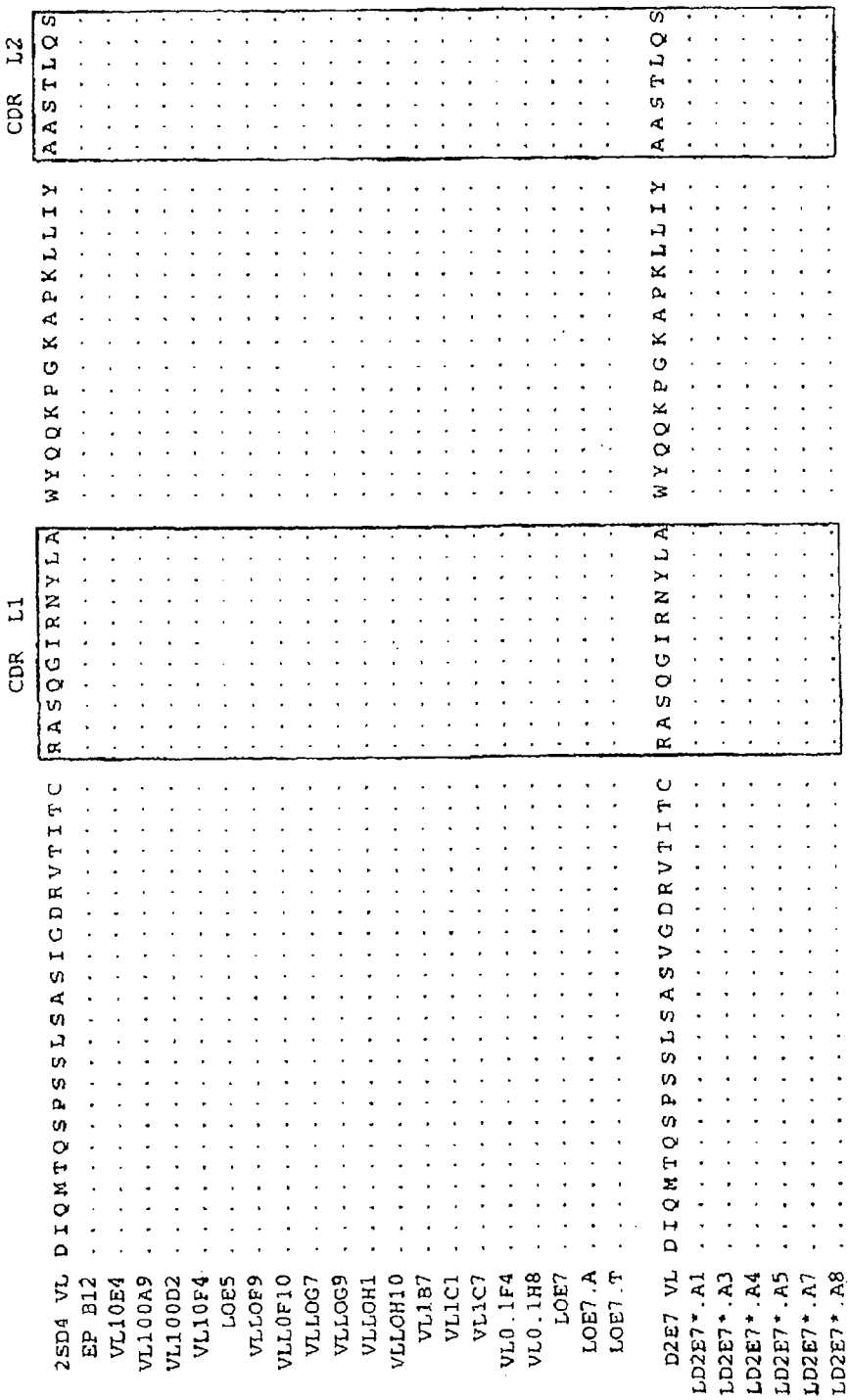
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CDR L2
2SD4 VL DIQMTQSPSSLSASIGDRVTITC RASQGIRNYLA WYQQKPGKAPKLLIY AASTLQS
EP B12
VL10E4
VL100A9
VL100D2
VL10F4
LOE5
VLLOF9
VLL0F10
VLLOG7
VLLOG9
VLLOH1
VLLOH10
VL1B7
VL1C1
VL1C7
VL0.1F4
VL0.1H8
LOE7
LOE7.A
LOE7.T
D2E7 VL DIQMTQSPSSLSASVGDRVTITC RASQGIRNYLA WYQQKPGKAPKLLIY AASTLQS
LD2E7*.A1
LD2E7*.A3
LD2E7*.A4
LD2E7*.A5
LD2E7*.A7
LD2E7*.A8

Figure 1A

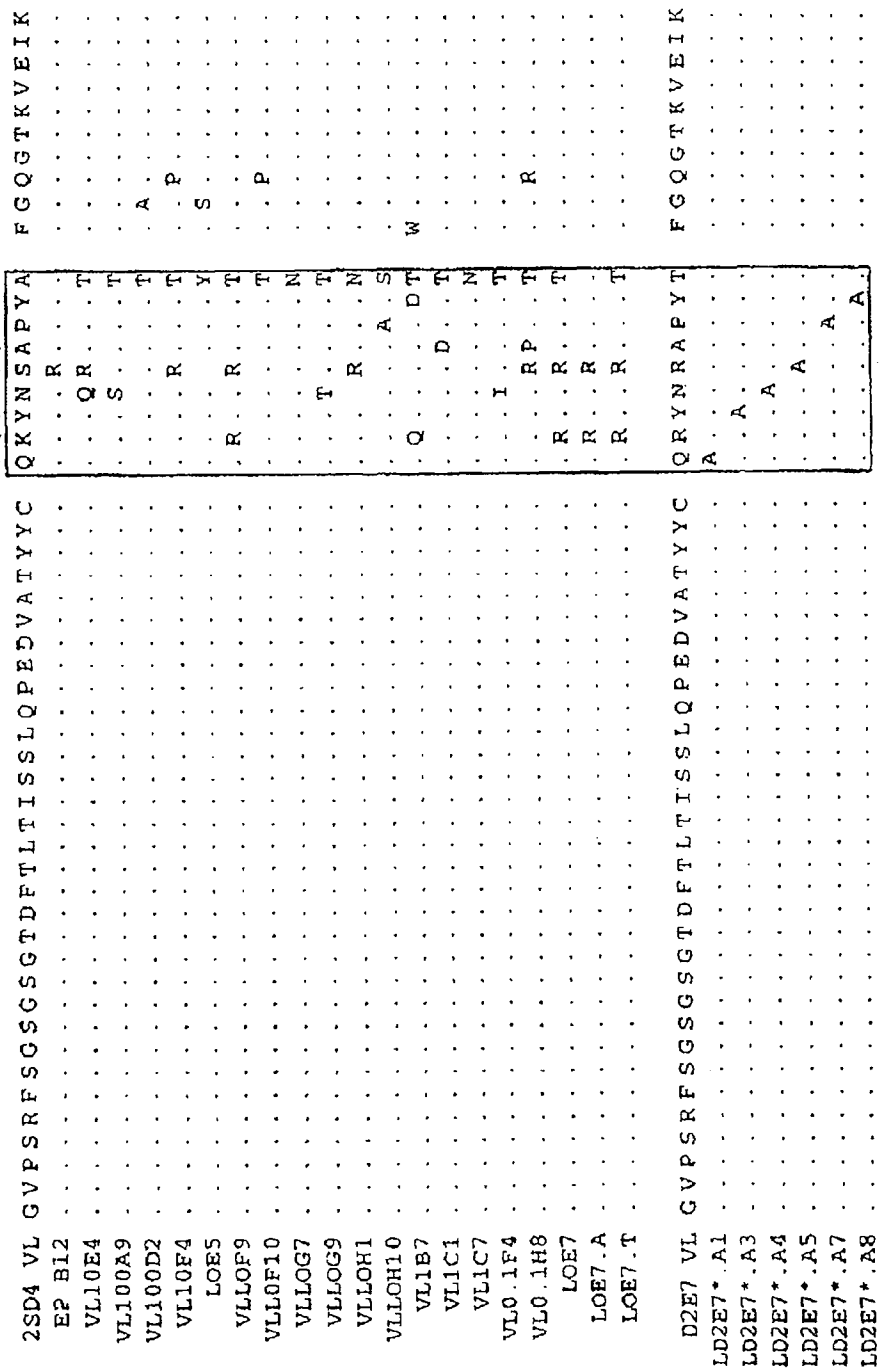

CDR L3
1 2 3 4 5 6 7 8 9
Q K Y N S A P Y A
F G Q G T K V E I K
2SD4 VL G V P S R F S G S G S G T D F T L T I S S L Q P E D V A T Y Y C
E2 B12
VL10E4
VL100A9
VL100D2
VL10F4
LOE5
VLLOF9
VLL0F10
VLLOG7
VLLOG9
VLLOH1
VLLOH10
VL1B7
VL1C1
VL1C7
VL0.1F4
VL0.1H8
LOE7
LOE7.A
LOE7.T
D2E7 VL G V P S R F S G S G S G T D F T L T I S S L Q P E D V A T Y Y C
Q R Y N R A P Y T
F G Q G T K V E I K
LD2E7*.A1
LD2E7*.A3
LD2E7*.A4
LD2E7*.A5
LD2E7*.A7
LD2E7*.A8

Figure 1B

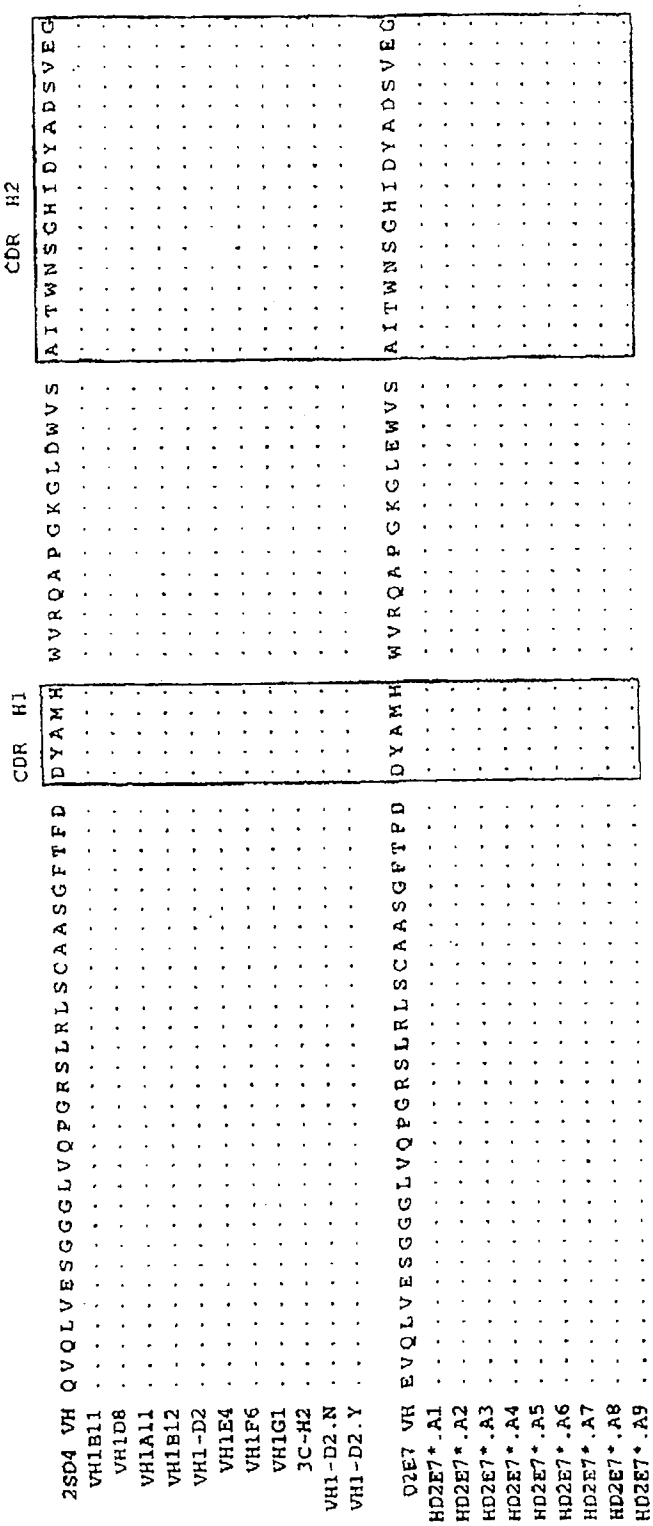

CDR H1
CDR H2
2SD4 VH QVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH WVRQAPGKGLDWVS AITWNSGHIDYADSVEG
VH1B11 . . . . . .
VH1D8 . . . . . .
VH1A11 . . . . . .
VH1B12 . . . . . .
VH1-D2 . . . . . .
VH1E4 . . . . . .
VH1F6 . . . . . .
VH1G1 . . . . . .
3C-H2 . . . . . .
VH1-D2.N . . . . . .
VH1-D2.Y . . . . . .
D2E7 VH EVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH WVRQAPGKGLEWVS AITWNSGHIDYADSVEG
HD2E7*.A1 . . . . . .
HD2E7*.A2 . . . . . .
HD2E7*.A3 . . . . . .
HD2E7*.A4 . . . . . .
HD2E7*.A5 . . . . . .
HD2E7*.A6 . . . . . .
HD2E7*.A7 . . . . . .
HD2E7*.A8 . . . . . .
HD2E7*.A9 . . . . . .

Figure 2A

CDR H3
1 2 3 4 5 6 7 8 9 10 11 12
2SD4 VH RFAVSRDNAKNALYLQMNSLRPEDTAVYYCTK ASYLSTSSSLDN WGQGTLVTVSS
VH1B11 K
VH1D8 AR Y
VH1A11 D
VH1B12 A F Y
VH1-D2 A V A Y
VH1E4 A H Y
VH1F6 A F E Y
VH1G1 A Y
3C-H2 A Y
VH1-D2.N A V A N
VH1-D2.Y A V A Y
D2E7 VH RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAK VSYLSTASSLDY WGQGTLVTVSS
HD2E7*.A1 A
HD2E7*.A2 A
HD2E7*.A3 A
HD2E7*.A4 A
HD2E7*.A5 A
HD2E7*.A6 A
HD2E7*.A7 A
HD2E7*.A8 A
HD2E7*.A9 A

Figure 2B

FIG. 3

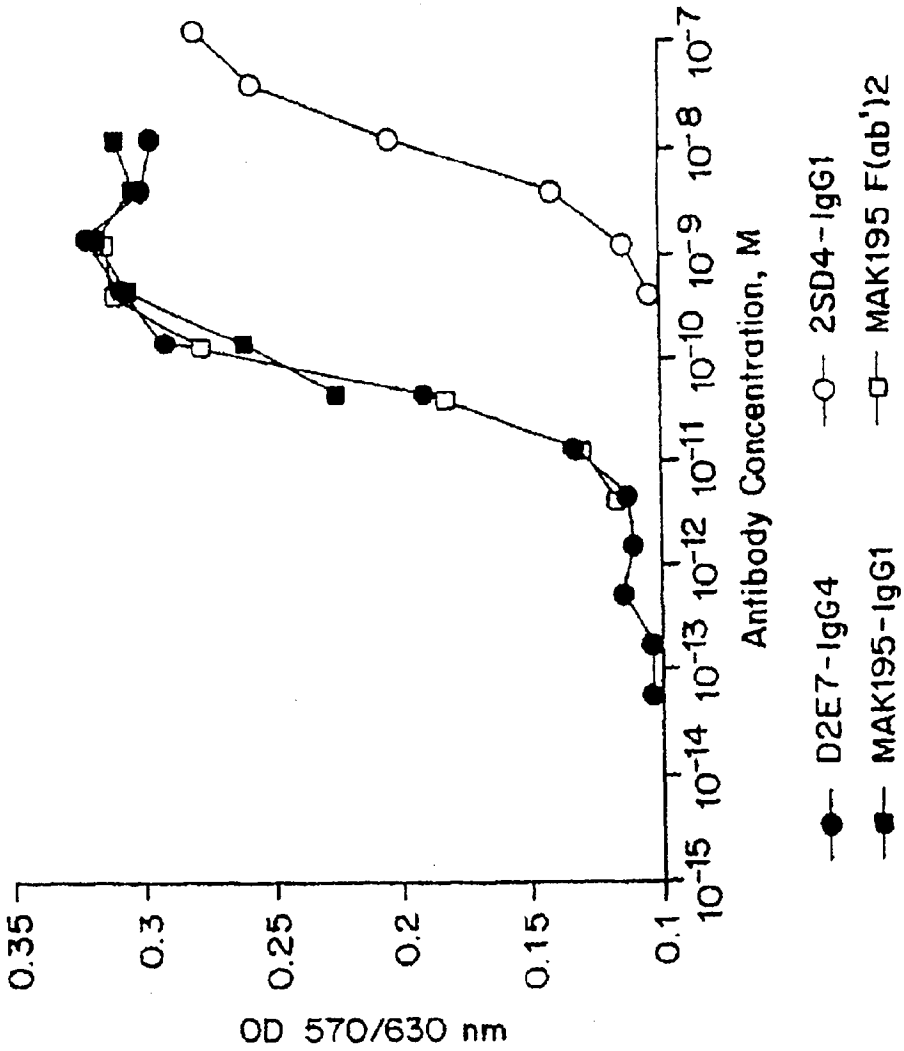

OD 570/630 nm
0.35
0.3
0.25
0.2
0.15
0.1
Antibody Concentration, M
10^{-15} 10^{-14} 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
D2E7-IgG4
MAK195-IgG1
2SD4-IgG1
MAK195 F(ab')2

FIG. 4

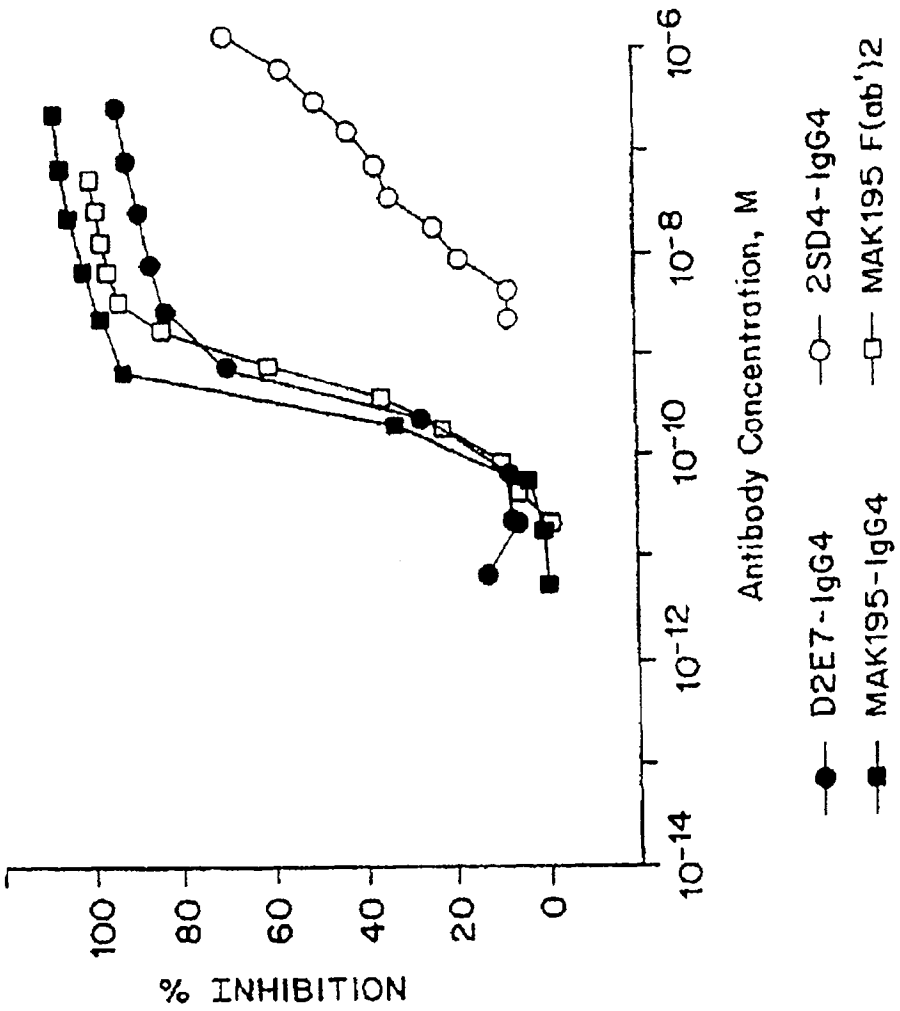

% INHIBITION
100
80
60
40
20
0
10^{-14}
10^{-12}
10^{-10}
10^{-8}
10^{-6}
Antibody Concentration, M
D2E7-IgG4
MAK195-IgG4
2SD4-IgG4
MAK195 F(ab')2

FIG. 5

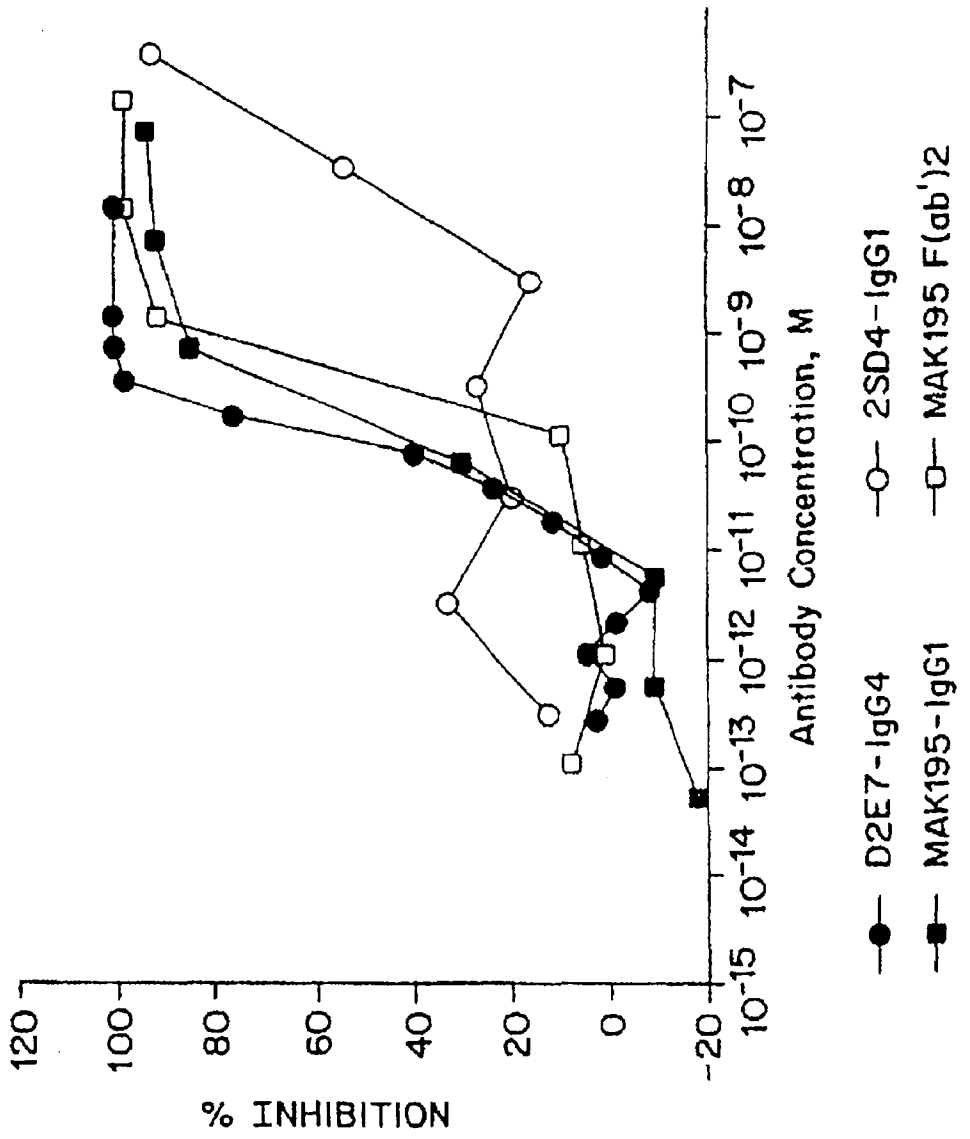
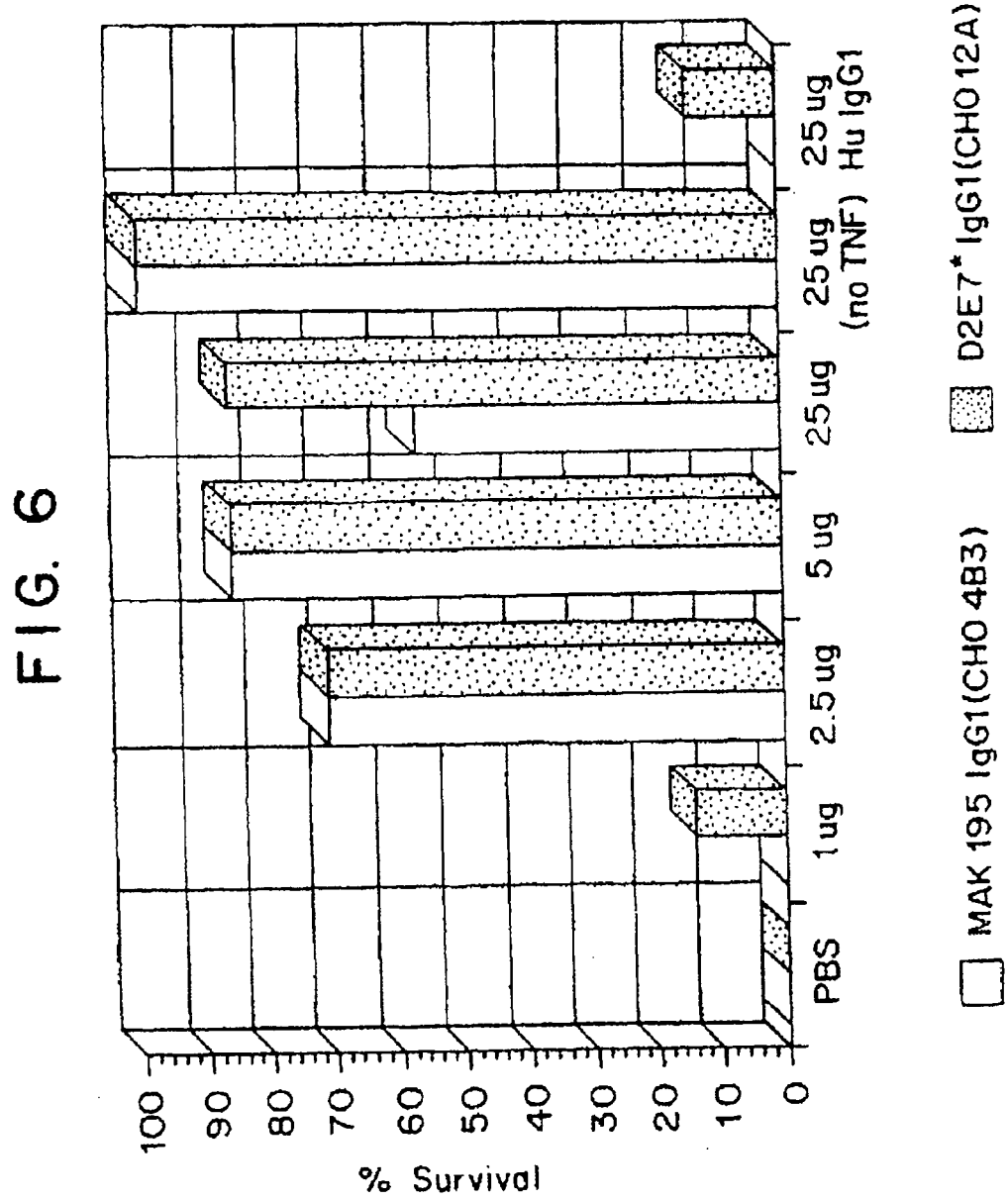

% INHIBITION
120
100
80
60
40
20
0
-20
Antibody Concentration, M
10^{-15} 10^{-14} 10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7}
D2E7-IgG4
2SD4-IgG1
MAK195-IgG1
MAK195 F(ab')2

FIG. 6


100
90
80
70
60
50
40
30
20
10
0
% Survival
PBS
1 ug
2.5 ug
5 ug
25 ug
25 ug (no TNF)
25 ug Hu IgG1
MAK 195 IgG1(CHO 4B3)
D2E7* IgG1(CHO 12A)

D2E7 VL

```
GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA
 D   I   Q   M   T   Q   S   P   S   S   L   S   A   S   V

                                                  CDR L1
GGG GAC AGA GTC ACC ATC ACT TGT CGG GCA AGT CAG GGC ATC AGA
 G   D   R   V   T   I   T   C   R   A   S   Q   G   I   R

AAT TAC TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG
 N   Y   L   A   W   Y   Q   Q   K   P   G   K   A   P   K

                                  CDR L2
CTC CTG ATC TAT GCT GCA TCC ACT TTG CAA TCA GGG GTC CCA TCT
 L   L   I   Y   A   A   S   T   L   Q   S   G   V   P   S

CGG TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC
 R   F   S   G   S   G   S   G   T   D   F   T   L   T   I

AGC AGC CTA CAG CCT GAA GAT GTT GCA ACT TAT TAC TGT CAA AGG
 S   S   L   Q   P   E   D   V   A   T   Y   Y   C   Q   R

  CDR L3
TAT AAC CGT GCA CCG TAT ACT TTT GGC CAG GGG ACC AAG GTG GAA
 Y   N   R   A   P   Y   T   F   G   Q   G   T   K   V   E

ATC AAA
 I   K
```

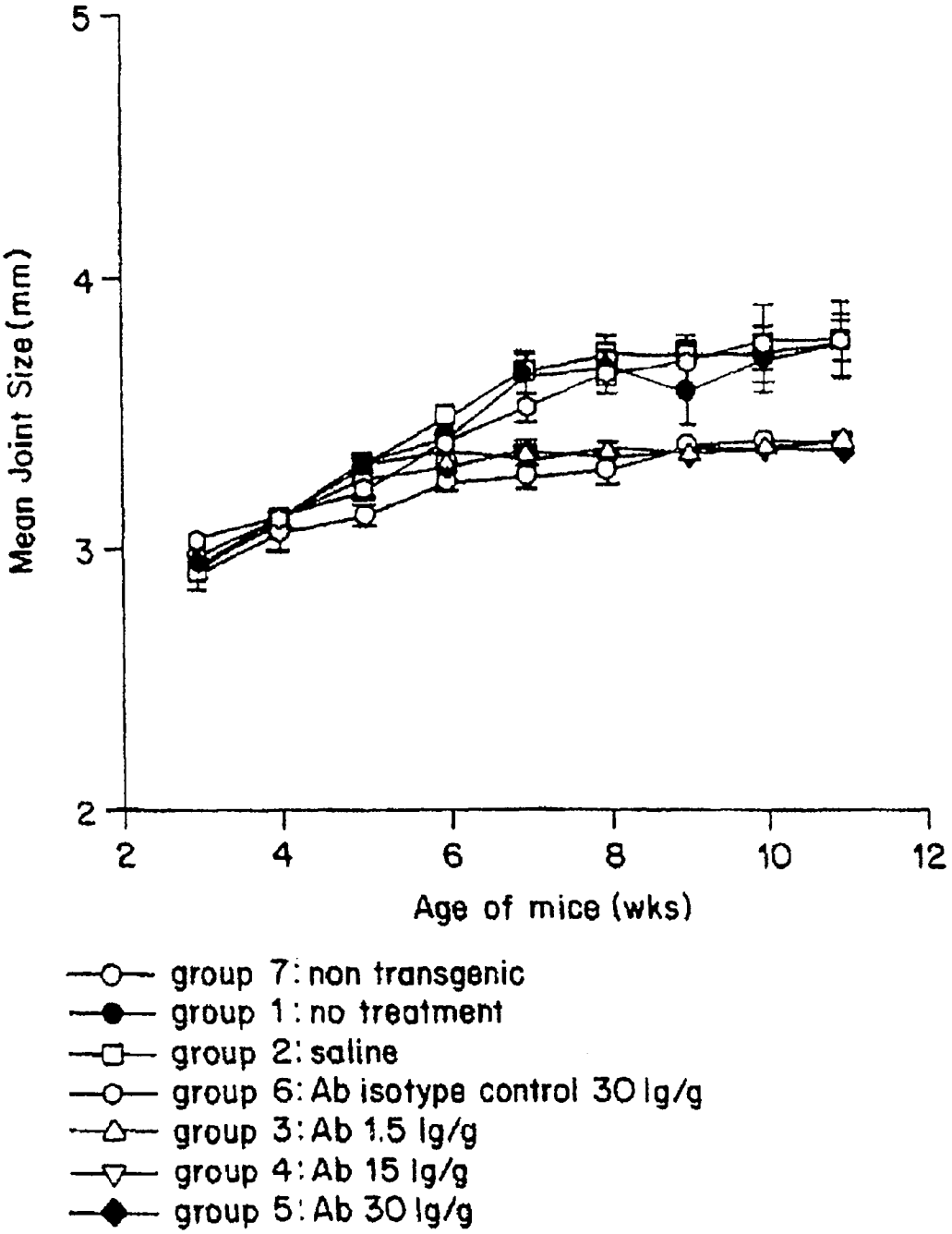
FIGURE 7

D2E7 VH

```
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC TTG GTA CAG CCC GGC
 E   V   Q   L   V   E   S   G   G   G   L   V   Q   P   G

AGG TCC CTG AGA CTC TCC TGT GCG GCC TCT GGA TTC ACC TTT GAT
 R   S   L   R   L   S   C   A   A   S   G   F   T   F   D

        CDR H1
GAT TAT GCC ATG CAC TGG GTC CGG CAA GCT CCA GGG AAG GGC CTG
 D   Y   A   M   H   W   V   R   Q   A   P   G   K   G   L

                                        CDR H2
GAA TGG GTC TCA GCT ATC ACT TGG AAT AGT GGT CAC ATA GAC TAT
 E   W   V   S   A   I   T   W   N   S   G   H   I   D   Y

GCG GAC TCT GTG GAG GGC CGA TTC ACC ATC TCC AGA GAC AAC GCC
 A   D   S   V   E   G   R   F   T   I   S   R   D   N   A

AAG AAC TCC CTG TAT CTG CAA ATG AAC AGT CTG AGA GCT GAG GAT
 K   N   S   L   Y   L   Q   M   N   S   L   R   A   E   D

                                                CDR H3
ACG GCC GTA TAT TAC TGT GCG AAA GTC TCG TAC CTT AGC ACC GCG
 T   A   V   Y   Y   C   A   K   V   S   Y   L   S   T   A

TCC TCC CTT GAC TAT TGG GGC CAA GGT ACC CTG GTC ACC GTC TCG
 S   S   L   D   Y   W   G   Q   G   T   L   V   T   V   S

AGT
 S
```

FIGURE 8

FIG. 9


Mean Joint Size (mm)
5
4
3
2
2
4
6
8
10
12
Age of mice (wks)
group 7: non transgenic
group 1: no treatment
group 2: saline
group 6: Ab isotype control 30 lg/g
group 3: Ab 1.5 lg/g
group 4: Ab 15 lg/g
group 5: Ab 30 lg/g

METHODS FOR TREATING RHEUMATOID ARTHRITIS USING HUMAN ANTIBODIES THAT BIND HUMAN TNFα

RELATED APPLICATIONS

This application is a continuation application of Ser. No. 09/801,185, filed on Mar. 7, 2001, which is a continuation of Ser. No. 09/125,098 filed on Mar. 16, 1999, now issued as U.S. Pat. No. 6,258,562, which claims priority to International Application Serial No. PCT/US97/02219 filed Feb. 10, 1997, which claims priority to U.S. provisional Application Ser. No. 60/031,476 filed Nov. 25, 1996. International Application Serial No. PCT/US97/02219 is also a continuation-in-part of U.S. application Ser. No. 08/599,226 filed Feb. 9, 1996. The contents of each of the above applications and patents are expressly incorporated by reference herein.

BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα) is a cytokine produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see e.g., Old, L. (1985) *Science* 230:630-632). Subsequently, a factor termed cachectin, associated with cachexia, was shown to be the same molecule as TNFα. TNFα has been implicated in mediating shock (see e.g., Beutler, B. and Cerami, A. (1988) *Annu. Rev. Biochem.* 57:505-518; Beutler, B. and Cerami, A. (1989) *Annu. Rev. Immunol.* 7:625-655). Furthermore, TNFα has been implicated in the pathophysiology of a variety of other human diseases and disorders, including sepsis, infections, autoimmune diseases, transplant rejection and graft-versus-host disease (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162-169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A., et al. Vasilli, P. (1992) *Annu. Rev. Immunol.* 10:411-452; Tracey, K. J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503).

Because of the harmful role of human TNFα (hTNFα) in a variety of human disorders, therapeutic strategies have been designed to inhibit or counteract hTNFα activity. In particular, antibodies that bind to, and neutralize, hTNFα have been sought as a means to inhibit hTNFα activity. Some of the earliest of such antibodies were mouse monoclonal antibodies (mAbs), secreted by hybridomas prepared from lymphocytes of mice immunized with hTNFα (see e.g., Hahn T; et al., (1985) *Proc Natl Acad Sci USA* 82: 3814-3818; Liang, C -M., et al. (1986) *Biochem. Biophys. Res. Commun.* 137:847-854; Hirai, M., et al. (1987) *J. Immunol. Methods* 96:57-62; Fendly, B. M., et al (1987) *Hybridoma* 6:359-370; Moeller, A., et al. (1990) *Cytokine* 2:162-169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 186 833 B1 by Wallach, D.; European Patent Application Publication No. 218 868 A1 by Old et al.; European Patent Publication No. 260 610 B1 by Moeller, A., et al.). While these mouse anti-hTNFα antibodies often displayed high affinity for hTNFα (e.g., Kd≦10^{-9}M) and were able to neutralize hTNFα activity, their use in vivo may be limited by problems associated with administration of mouse antibodies to humans, such as short serum half life, an inability to trigger certain human effector functions and elicitation of an unwanted immune response against the mouse antibody in a human (the "human anti-mouse antibody" (HAMA) reaction).

In an attempt to overcome the problems associated with use of fully-murine antibodies in humans, murine anti-hTNFα antibodies have been genetically engineered to be more "human-like." For example, chimeric antibodies, in which the variable regions of the antibody chains are murine-derived and the constant regions of the antibody chains are human-derived, have been prepared (Knight, D. M, et al. (1993) *Mol. Immunol.* 30:1443-1453; PCT Publication No. WO 92/16553 by Daddona, P. E., et al.). Additionally, humanized antibodies, in which the hypervariable domains of the antibody variable regions are murine-derived but the remainder of the variable regions and the antibody constant regions are human-derived, have also been prepared (PCT Publication No. WO 92/11383 by Adair, J. R., et al.). However, because these chimeric and humanized antibodies still retain some murine sequences, they still may elicit an unwanted immune reaction, the human anti-chimeric antibody (HACA) reaction, especially when administered for prolonged periods, e.g., for chronic indications, such as rheumatoid arthritis (see e.g., Elliott, M. J., et al. (1994) *Lancet* 344:1125-1127; Elliot, M. J., et al. (1994) *Lancet* 344:1105-1110).

A preferred hTNFα inhibitory agent to murine mAbs or derivatives thereof (e.g., chimeric or humanized antibodies) would be an entirely human anti-hTNFα antibody, since such an agent should not elicit the HAMA reaction, even if used for prolonged periods. Human monoclonal autoantibodies against hTNFα have been prepared using human hybridoma techniques (Boyle, P., et al. (1993) *Cell. Immunol.* 152:556-568; Boyle, P., et al. (1993) *Cell. Immunol.* 152:569-581; European Patent Application Publication No. 614 984 A2 by Boyle, et al.). However, these hybridoma-derived monoclonal autoantibodies were reported to have an affinity for hTNFα that was too low to calculate by conventional methods, were unable to bind soluble hTNFα and were unable to neutralize hTNFα-induced cytotoxicity (see Boyle, et al.; supra). Moreover, the success of the human hybridoma technique depends upon the natural presence in human peripheral blood of lymphocytes producing autoantibodies specific for hTNFα. Certain studies have detected serum autoantibodies against hTNFα in human subjects (Fomsgaard, A., et al. (1989) *Scand. J Immunol.* 30:219-223; Bendtzen, K., et al. (1990) *Prog. Leukocyte Biol.* 10B:447-452), whereas others have not (Leusch, H -G., et al. (1991) *J. Immunol. Methods* 139:145-147).

Alternative to naturally-occurring human anti-hTNFα antibodies would be a recombinant hTNFα antibody. Recombinant human antibodies that bind hTNFα with relatively low affinity (i.e., K_d~10^{-7}M) and a fast off rate (i.e., K_{off}~10^{-2} sec^{-1}) have been described (Griffiths, A. D., et al. (1993) *EMBO J.* 12:725-734). However, because of their relatively fast dissociation kinetics, these antibodies may not be suitable for therapeutic use. Additionally, a recombinant human anti-hTNFα has been described that does not neutralize hTNFα activity, but rather enhances binding of hTNFα to the surface of cells and enhances internalization of hTNFα (Lidbury, A., et al. (1994) *Biotechnol. Ther.* 5:27-45; PCT Publication No. WO 92/03145 by Aston, R. et al.)

Accordingly, human antibodies, such as recombinant human antibodies, that bind soluble hTNFα with high affinity and slow dissociation kinetics and that have the capacity to neutralize hTNFα activity, including hTNFα-induced cytotoxicity (in vitro and in vivo) and hTNFα-induced cell activation, are still needed.

SUMMARY OF THE INVENTION

This invention provides human antibodies, preferably recombinant human antibodies, that specifically bind to human TNFα. The antibodies of the invention are characterized by binding to hTNFα with high affinity and slow dissociation kinetics and by neutralizing hTNFα activity, includ-

ing hTNFα-induced cytotoxicity (in vitro and in vivo) and hTNFα-induced cellular activation. Antibodies of the invention are further characterized by binding to hTNFα but not hTNFβ (lymphotoxin) and by having the ability to bind to other primate TNFαs and non-primate TNFαs in addition to human TNFα.

The antibodies of the invention can be full-length (e.g., an IgG1 or IgG4 antibody) or can comprise only an antigen-binding portion (e.g., a Fab, $F(ab')_2$ or scFv fragment). The most preferred recombinant antibody of the invention, termed D2E7, has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 and a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4. Preferably, the D2E7 antibody has a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2.

In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a K_{off} of 1×10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10^{-9} M or less and still more preferably with an IC_{50} of 5×10^{-10} M or less.

In another embodiment, the invention provides a human antibody, or antigen-binding portion thereof, with the following characteristics:

a) dissociates from human TNFα with a K_{off} of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less. Still more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 1×10^{-4} s^{-1} or less.

In yet another embodiment, the invention provides a human antibody, or an antigen-binding portion thereof, with an LCVR having CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with an HCVR having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. More preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6. Still more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with an LCVR comprising the amino acid sequence of SEQ ID NO: 1 and an HCVR comprising the amino acid sequence of SEQ ID NO: 2. In certain embodiments, the antibody has an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. In yet other embodiments, the antibody is a Fab fragment, an $F(ab')_2$ fragment or a single chain Fv fragment.

In still other embodiments, the invention provides antibodies, or antigen-binding portions thereof, with an LCVR having CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26 or with an HCVR having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, that neutralizes the activity of human TNFα but not human TNFβ (lymphotoxin). In a preferred embodiment, the human antibody, or antigen-binding portion thereof, neutralizes the activity of human TNFα, chimpanzee TNFα and at least one additional primate TNFα selected from the group consisting of baboon TNFα, marmoset TNFα, cynomolgus TNFα and rhesus TNFα. Preferably, the antibody also neutralizes the activity of at least one non-primate TNFα. For example, in one subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of canine TNFα. In another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of pig TNFα. In yet another subembodiment, the isolated human antibody, or antigen-binding portion thereof, also neutralizes the activity of mouse TNFα.

Another aspect of the invention pertains to nucleic acid molecules encoding the antibodies, or antigen-binding portions, of the invention. A preferred nucleic acid of the invention, encoding a D2E7 LCVR, has the nucleotide sequence shown in FIG. 7 and SEQ ID NO 36. Another preferred nucleic acid of the invention, encoding a D2E7 HCVR, has the nucleotide sequence shown in FIG. 8 and SEQ ID NO 37. Recombinant expression vectors carrying the antibody-encoding nucleic acids of the invention, and host cells into which such vectors have been introduced, are also encompassed by the invention, as are methods of making the antibodies of the invention by culturing the host cells of the invention.

Yet another aspect of the invention pertains to methods for inhibiting human TNFα activity using an antibody, or antigen-binding portion thereof, of the invention. In one embodiment, the method comprises contacting human TNFα with the antibody of the invention, or antigen-binding portion thereof, such that human TNFα activity is inhibited. In another embodiment, the method comprises administering an antibody of the invention, or antigen-binding portion thereof, to a human subject suffering from a disorder in which TNFα activity is detrimental such that human TNFα activity in the human subject is inhibited. The disorder can be, for example,

sepsis, an autoimmune disease (e.g., rheumatoid arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome), an infectious disease, a malignancy, transplant rejection or graft-versus-host disease, a pulmonary disorder, a bone disorder, an intestinal disorder or a cardiac disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and 1B show the amino acid sequences of the light chain variable region of D2E7 (D2E7 VL; also shown in SEQ ID NO: 1), alanine-scan mutants of D2E7 VL (LD2E7*.A1, LD2E7*.A3, LD2E7*.A4, LD2E7*.A5, LD2E7*.A7 and LD2E7*.A8), the light chain variable region of the D2E7-related antibody 2SD4 (2SD4 VL; also shown in SEQ ID NO: 9) and other D2E7-related light chain variable regions (EP B12, VL10E4, VL100A9, VL100D2, VL10F4, LOE5, VLLOF9, VLL0F10, VLLOG7, VLLOG9, VLLOH1, VLLOH10, VL1B7, VL1C1, VL1C7, VL0.1F4, VL0.1H8, LOE7, LOE7.A and LOE7.T). FIG. **1**A shows the FR1, CDR1, FR2 and CDR2 domains. FIG. **1**B shows the FR3, CDR3 and FR4 domains. The light chain CDR1 ("CDR L1"), CDR2 ("CDR L2") and CDR3 ("CDR L3") domains are boxed.

FIGS. **2**A and 2B show the amino acid sequences of the heavy chain variable region of D2E7 (D2E7 VH; also shown in SEQ ID NO: 2), alanine-scan mutants of D2E7 VH (HD2E7*.A1, HD2E7*.A2, HD2E7*.A3, HD2E7*.A4, HD2E7*.A5, HD2E7*.A6, HD2E7*.A7, HD2E7*.A8 and HD2E7*.A9), the heavy chain variable region of the D2E7-related antibody 2SD4 (2SD4 VH; also shown in SEQ ID NO: 10) and other D2E7-related heavy chain variable regions (VH1B11, VH1D8, VH1A11, VH1B12, VH1-D2, VH1E4, VH1F6, VH1G1, 3C—H2, VH1-D2.N and VH1-D2.Y). FIG. **2**A shows the FR1, CDR1, FR2 and CDR2 domains. FIG. **2**B shows the FR3, CDR3 and FR4 domains. The heavy chain CDR1 ("CDR H1"), CDR2 ("CDR H2") and CDR3 ("CDR H3") domains are boxed.

FIG. **3** is a graph depicting the inhibition of TNFα-induced L929 cytotoxicity by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. **4** is a graph depicting the inhibition of rhTNFα binding to hTNFα receptors on U-937 cells by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. **5** is a graph depicting the inhibition of TNFα-induced ELAM-1 expression on HVEC by the human anti-hTNFα antibody D2E7, as compared to the murine anti-hTNFα antibody MAK 195.

FIG. **6** is a bar graph depicting protection from TNFα-induced lethality in D-galactosamine-sensitized mice by administration of the human anti-hTNFα antibody D2E7 (black bars), as compared to the murine anti-hTNFα antibody MAK 195 (hatched bars).

FIG. **7** shows the nucleotide sequence of the light chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR L1, CDR L2 and CDR L3 regions are underlined.

FIG. **8** shows the nucleotide sequence of the heavy chain variable region of D2E7, with the predicted amino acid sequence below the nucleotide sequence. The CDR H1, CDR H2 and CDR H3 regions are underlined.

FIG. **9** is a graph depicting the effect of D2E7 antibody treatment on the mean joint size of Tg197 transgenic mice as a polyarthritis model.

DETAILED DESCRIPTION OF THE INVENTION

This invention pertains to isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Various aspects of the invention relate to antibodies and antibody fragments, and pharmaceutical compositions thereof, as well as nucleic acids, recombinant expression vectors and host cells for making such antibodies and fragments. Methods of using the antibodies of the invention to detect human TNFα or to inhibit human TNFα activity, either in vitro or in vivo, are also encompassed by the invention.

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNFα" (abbreviated herein as hTNFα, or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNFα is described further in, for example, Pennica, D., et al. (1984) *Nature* 312:724-729; Davis, J. M., et al. (1987) *Biochemistry* 26:1322-1326; and Jones, E. Y., et al. (1989) *Nature* 338:225-228. The term human TNFα is intended to include recombinant human TNFα (rhTNFα), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, Minn.).

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes,

they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) *Science* 242:423-426; and Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R. J., et al. (1994) *Structure* 2:1121-1123).

Still further, an antibody or antigen-binding portion thereof may be part of a larger immunoadhesion molecules, formed by covalent or noncovalent association of the antibody or antibody portion with one or more other proteins or peptides. Examples of such immunoadhesion molecules include use of the streptavidin core region to make a tetrameric scFv molecule (Kipriyanov, S. M., et al. (1995) *Human Antibodies and Hybridomas* 6:93-101) and use of a cysteine residue, a marker peptide and a C-terminal polyhistidine tag to make bivalent and biotinylated scFv molecules (Kipriyanov, S. M., et al. (1994) *Mol. Immunol.* 31:1047-105 8). Antibody portions, such as Fab and $F(ab')_2$ fragments, can be prepared from whole antibodies using conventional techniques, such as papain or pepsin digestion, respectively, of whole antibodies. Moreover, antibodies, antibody portions and immunoadhesion molecules can be obtained using standard recombinant DNA techniques, as described herein.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further in Section II, below), antibodies isolated from a recombinant, combinatorial human antibody library (described further in Section III, below), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor, L. D., et al. (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to in vitro mutagenesis (or, when an animal transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

An "isolated antibody", as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα. An isolated antibody that specifically binds hTNFα may, however, have cross-reactivity to other antigens, such as TNFα molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody", as used herein (or an "antibody that neutralized hTNFα activity"), is intended to refer to an antibody whose binding to hTNFα results in inhibition of the biological activity of hTNFα. This inhibition of the biological activity of hTNFα can be assessed by measuring one or more indicators of hTNFα biological activity, such as hTNFα-induced cytotoxicity (either in vitro or in vivo), hTNFα-induced cellular activation and hTNFα binding to hTNFα receptors. These indicators of hTNFα biological activity can be assessed by one or more of several standard in vitro or in vivo assays known in the art (see Example 4). Preferably, the ability of an antibody to neutralize hTNFα activity is assessed by inhibition of hTNFα-induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNFα activity, the ability of an antibody to inhibit hTNFα-induced expression of ELAM-1 on HUVEC, as a measure of hTNFα-induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, N.J.). For further descriptions, see Example 1 and Jönsson, U., et al. (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., et al. (1991) *Biotechniques* 11:620-627; Johnsson, B., et al. (1995) *J. Mol. Recognit.* 8:125-131; and Johnnson, B., et al. (1991) *Anal. Biochem.* 198:268-277.

The term "K_{off}", as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_d", as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule", as used herein in reference to nucleic acids encoding antibodies or antibody portions (e.g., VH, VL, CDR3) that bind hTNFα, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-TNFα antibody contains no other sequences encoding other VH regions that bind antigens other than TNFα.

The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA

loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

Various aspects of the invention are described in further detail in the following subsections.

I. Human Antibodies that Bind Human TNFα

This invention provides isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNFα antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 and has VL and VH sequences as shown in FIGS. 1A, 1B and FIG. 2A, 2B, respectively (the amino acid sequence of the D2E7 VL region is also shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is also shown in SEQ ID NO: 2). The binding properties of D2E7, as compared to the murine anti-hTNFα MAK 195 mAb that exhibits high affinity and slow dissociation kinetics and another human anti-hTNFα antibody related in sequence to D2E7, 2SD4, are summarized below:

Antibody	K_{off} sec^{-1}	K_{on} $M^{-1}sec^{-1}$	K_d M	Stoichiometry
D2E7 IgG1	8.81×10^{-5}	1.91×10^{5}	6.09×10^{-10}	1.2
2SD4 IgG4	8.4×10^{-3}	4.20×10^{5}	2.00×10^{-8}	0.8
MAK 195 $F(ab')_2$	8.70×10^{-5}	1.90×10^{5}	4.60×10^{-10}	1.4

The D2E7 antibody, and related antibodies, also exhibit a strong capacity to neutralize hTNFα activity, as assessed by several in vitro and in vivo assays (see Example 4). For example, these antibodies neutralize hTNFα-induced cytotoxicity of L929 cells with IC_{50} values in the range of about 10^{-7} M to about 10^{-10} M. D2E7, when expressed as a full-length IgG1 antibody, neutralizes hTNFα-induced cytotoxicity of L929 cells with IC_{50} of about 1.25×10^{-10} M. Moreover, the neutralizing capacity of D2E7 is maintained when the antibody is expressed as a Fab, $F(ab')_2$ or scFv fragment. D2E7 also inhibits TNFα-induced cellular activation, as measured by hTNFα-induced ELAM-1 expression on HUVEC (IC_{50}=about 1.85×10^{-10} M), and binding of hTNFα to hTNFα receptors on U-937 cells (IC_{50}=about 1.56×10^{-10} M). Regarding the latter, D2E7 inhibits the binding of hTNFα to both the p55 and p75 hTNFα receptors. Furthermore, the antibody inhibits hTNFα-induced lethality in vivo in mice (ED_{50}=1-2.5 μg/mouse).

Regarding the binding specificity of D2E7, this antibody binds to human TNFα in various form, including soluble hTNFα, transmembrane hTNFα and hTNFα bound to cellular receptors. D2E7 does not specifically bind to other cytokines, such as lymphotoxin (TNFβ), IL-1αa:, IL-1β, IL-2, IL-4, IL-6, IL-8, IFNγ and TGFβ. However, D2E7 does exhibit crossreactivity to tumor necrosis factors from other species. For example, the antibody neutralizes the activity of at least five primate TNFαs (chimpanzee, baboon, marmoset, cynomolgus and rhesus) with approximately equivalent IC_{50} values as for neutralization of hTNFα (see Example 4, subsection E). D2E7 also neutralizes the activity of mouse TNFα, although approximately 1000-fold less well than human TNFα (see Example 4, subsection E). D2E7 also binds to canine and porcine TNFα.

In one aspect, the invention pertains to D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a K_{off} of 1×10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10–9 M or less and still more preferably with an IC_{50} of 5×10–10 M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof. In another preferred embodiment, the antibody also neutralizes TNFα-induced cellular activation, as assessed using a standard in vitro assay for TNFα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC).

Surface plasmon resonance analysis for determining K_d and K_{off} can be performed as described in Example 1. A standard in vitro L929 assay for determining IC_{50} values is described in Example 4, subsection A. A standard in vitro assay for TNFα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC) is described in Example 4, subsection C. Examples of recombinant human antibodies that meet, or are predicted to meet, the aforementioned kinetic and neutralization criteria include antibodies having the following [VH/VL] pairs, the sequences of which are shown in FIGS. 1A, 1B, 2A and 2B (see also Examples 2, 3 and 4 for kinetic and neutralization analyses): [D2E7 VH/D2E7 VL]; [HD2E7*.A1/D2E7 VL], [HD2E7*.A2/D2E7 VL], [HD2E7*.A3/D2E7 VL], [HD2E7*.A4/D2E7

VL], [HD2E7*.A5/D2E7 VL], [HD2E7*.A6/D2E7 VL], [HD2E7*.A7/D2E7 VL], [HD2E7*.A8/D2E7 VL], [HD2E7*.A9/D2E7 VL], [D2E7 VH/LD2E7*.A1], [D2E7 VH/LD2E7*.A4], [D2E7 VH/LD2E7*.A5], [D2E7 VH/LD2E7*.A7], [D2E7 VH/LD2E7*.A8], [HD2E7*.A9/LD2E7*.A1],[VH1-D2/LOE7], [VH1-D2.N/LOE7.T], [VH1-D2.Y/LOE7.A], [VH1-D2.N/LOE7.A], [VH1-D2/EP B12] and [3C—H2/LOE7].

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to human antibodies that have slow dissociation kinetics for association with hTNFα and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. As demonstrated in Example 3, position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R—Y—N—R-A-P—Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V—S—Y-L-S—T-A-S—S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off}. Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNFα. As shown in Example 3, positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNFα and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above).

Accordingly, in another embodiment, the invention provides an isolated human antibody, or antigen-binding portion thereof, with the following characteristics:

a) dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 1×10^{-4} s^{-1} or less.

In yet another embodiment, the invention provides an isolated human antibody, or an antigen-binding portion thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (i.e., the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (i.e., the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (i.e., the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (i.e., the D2E7 VH CDR1). The framework regions for VL preferably are from the $V_{\kappa}I$ human germline family, more preferably from the A20 human germline Vk gene and most preferably from the D2E7 VL framework sequences shown in FIGS. 1A and 1B. The framework regions for VH preferably are from the V_H3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in FIGS. 2A and 2B.

In still another embodiment, the invention provides an isolated human antibody, or an antigen binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides an isolated human antibody, or an antigen-binding portions thereof, having D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID

NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32. SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In yet another embodiment, the invention provides a recombinant human antibody, or antigen-binding portion thereof, that neutralizes the activity of human TNFα but not human TNFβ. Preferably, antibody, or antigen-binding portion thereof, also neutralizes the activity of chimpanzee TNFα and at least one additional primate TNFα selected from the group consisting of baboon TNFα, marmoset TNFα, cynomolgus TNFα and rhesus TNFα. Preferably, the antibody, or antigen-binding portion thereof, neutralizes the human, chimpanzee and/or additional primate TNFα in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less, more preferably 1×10^{-9} M or less, and even more preferably 5×10^{-10} M or less. In one subembodiment, the antibody also neutralizes the activity of canine TNFα, preferably in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less, more preferably 1×10^{-8} M or less and even more preferably 5×10^{-9} M or less. In another subembodiment, the antibody also neutralizes the activity of pig TNFα, preferably with an IC_{50} of 1×10^{-5} M or less, more preferably 1×10^{-6} M or less and even more preferably 5×10^{-7} M or less. In yet another embodiment, the antibody also neutralizes the activity of mouse TNFα, preferably with an IC_{50} of 1×10^{-4} M or less, more preferably 1×10^{-5} M or less and even more preferably 5×10^{-6} M or less.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (e.g., another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNFα antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (e.g., a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, e.g., to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (e.g., disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, Ill.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

II. Expression of Antibodies

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F. M. et al. (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Pat. No. 4,816,397 by Boss et al.

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see e.g., the "Vbase" human germline sequence database; see also Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., et al. (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J. P. L. et al. (1994) "A Directory of Human Germ-line V_κ Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the $V_\kappa I$ family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-3 1 germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited supra, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino

acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Moreover, it should be noted that if the "germline" sequences obtained by PCR amplification encode amino acid differences in the framework regions from the true germline configuration (i.e., differences in the amplified sequence as compared to the true germline sequence, for example as a result of somatic mutation), it may be desireable to change these amino acid differences back to the true germline sequences (i.e., "backmutation" of framework residues to the germline configuration).

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see e.g., Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat, E. A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, e.g., encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see e.g., Bird et al. (1988) *Science* 242:423-426; Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty et al., *Nature* (1990) 348:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (e.g., the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see e.g., U.S. Pat. No. 5,168,062 by Stinski, U.S. Pat. No. 4,510,245 by Bell et al. and U.S. Pat. No. 4,968,615 by Schaffner et al.

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that

regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., U.S. Pat. Nos. 4,399, 216, 4,634,665 and 5,179,017, all by Axel et al.). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M. A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufmnan and P. A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNFα. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNFα by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

In view of the foregoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of the antibodies and antibody portions of the invention. The nucleotide sequence encoding the D2E7 light chain variable region is shown in FIG. 7 and SEQ ID NO: 36. The CDR1 domain of the LCVR encompasses nucleotides 70-102, the CDR2 domain encompasses nucleotides 148-168 and the CDR3 domain encompasses nucleotides 265-291. The nucleotide sequence encoding the D2E7 heavy chain variable region is shown in FIG. 8 and SEQ ID NO: 37. The CDR1 domain of the HCVR encompasses nucleotides 91-105, the CDR2 domain encompasses nucleotides 148-198 and the CDR3 domain encompasses nucleotides 295-330. It will be appreciated by the skilled artisan that nucleotide sequences encoding D2E7-related antibodies, or portions thereof (e.g., a CDR domain, such as a CDR3 domain), can be derived from the nucleotide sequences encoding the D2E7 LCVR and HCVR using the genetic code and standard molecular biology techniques.

In one embodiment, the invention provides an isolated nucleic acid encoding a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3 (i.e., the D2E7 VL CDR3), or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9. This nucleic acid can encode only the CDR3 region or, more preferably, encodes an entire antibody light chain variable region (LCVR). For example, the nucleic acid can encode an LCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (i.e., the D2E7 VL CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (i.e., the D2E7 VL CDR1).

In another embodiment, the invention provides an isolated nucleic acid encoding a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4 (i.e., the D2E7 VH CDR3), or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12. This nucleic acid can encode only the CDR3 region or, more preferably, encodes an entire antibody heavy chain variable region (HCVR). For example, the nucleic acid can encode a HCVR having a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (i.e., the D2E7 VH CDR2) and a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (i.e., the D2E7 VH CDR1).

In yet another embodiment, the invention provides isolated nucleic acids encoding a D2E7-related CDR3 domain, e.g., comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 3, SEQ ID NO 4, SEQ ID NO: 11,

SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody light chain variable region comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 LCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 36, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 1. The nucleic acid can encode only the LCVR or can also encode an antibody light chain constant region, operatively linked to the LCVR. In one embodiment, this nucleic acid is in a recombinant expression vector.

In still another embodiment, the invention provides an isolated nucleic acid encoding an antibody heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 HCVR). Preferably this nucleic acid comprises the nucleotide sequence of SEQ ID NO: 37, although the skilled artisan will appreciate that due to the degeneracy of the genetic code, other nucleotide sequences can encode the amino acid sequence of SEQ ID NO: 2. The nucleic acid can encode only the HCVR or can also encode a heavy chain constant region, operatively linked to the HCVR. For example, the nucleic acid can comprise an IgG1 or IgG4 constant region. In one embodiment, this nucleic acid is in a recombinant expression vector.

The invention also provides recombinant expression vectors encoding both an antibody heavy chain and an antibody light chain. For example, in one embodiment, the invention provides a recombinant expression vector encoding:

a) an antibody light chain having a variable region comprising the amino acid sequence of SEQ ID NO: 1 (i.e., the D2E7 LCVR); and

b) an antibody heavy chain having a variable region comprising the amino acid sequence of SEQ ID NO: 2 (i.e., the D2E7 HCVR).

The invention also provides host cells into which one or more of the recombinant expression vectors of the invention have been introduced. Preferably, the host cell is a mammalian host cell, more preferably the host cell is a CHO cell, an NS0 cell or a COS cell.

Still further the invention provides a method of synthesizing a recombinant human antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant human antibody of the invention is synthesized. The method can further comprise isolating the recombinant human antibody from the culture medium.

III. Selection of Recombinant Human Antibodies

Recombinant human antibodies of the invention in addition to the D2E7 or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from MRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene SurfZAP™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Ladner et al. U.S. Pat. No. 5,223,409; Kang et al. PCT Publication No. WO 92/18619; Dower et al. PCT Publication No. WO 91/17271; Winter et al. PCT Publication No. WO 92/20791; Markland et al PCT Publication No. WO 92/15679; Breitling et al. PCT Publication No. WO 93/01288; McCafferty et al. PCT Publication No. WO 92/01047; Garrard et al. PCT Publication No. WO 92/09690; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; McCafferty et al., *Nature* (1990) 348:552-554; Griffiths et al. (1993) *EMBO J.* 12:725-734; Hawkins et al. (1992) *J. Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In a preferred embodiment, to isolate human antibodies with high affinity and a low off rate constant for hTNFα, a murine anti-hTNFα antibody having high affinity and a low off rate constant for hTNFα (e.g., MAK 195, the hybridoma for which has deposit number ECACC 87 050801) is first used to select human heavy and light chain sequences having similar binding activity toward hTNFα, using the epitope imprinting, or guided selection, methods described in Hoogenboom et al., PCT Publication No. WO 93/06213. The antibody libraries used in this method are preferably scfv libraries prepared and screened as described in McCafferty et al., PCT Publication No. WO 92/01047, McCafferty et al., *Nature* (1990) 348:552-554; and Griffiths et al., (1993) *EMBO J.* 12:725-734. The scFv antibody libraries preferably are screened using recombinant human TNFα as the antigen.

Once initial human VL and VH segments are selected, "mix and match" experiments, in which different pairs of the initially selected VL and VH segments are screened for hTNFα binding, are performed to select preferred VL/VH pair combinations. Additionally, to further improve the affinity and/or lower the off rate constant for hTNFα binding, the VL and VH segments of the preferred VL/VH pair(s) can be randomly mutated, preferably within the CDR3 region of VH and/or VL, in a process analogous to the in vivo somatic mutation process responsible for affinity maturation of antibodies during a natural immune response. This in vitro affinity maturation can be accomplished by amplifying VH and VL regions using PCR primers complimentary to the VH CDR3 or VL CDR3, respectively, which primers have been "spiked" with a random mixture of the four nucleotide bases at certain positions such that the resultant PCR products encode VH and VL segments into which random mutations have been introduced into the VH and/or VL CDR3 regions. These randomly mutated VH and VL segments can be rescreened for binding to hTNFα and sequences that exhibit high affinity and a low off rate for hTNFα binding can be selected.

The amino acid sequences of selected antibody heavy and light chains can be compared to germline heavy and light chain amino acid sequences. In cases where certain framework residues of the selected VL and/or VH chains differ from the germline configuration (e.g., as a result of somatic mutation of the immunoglobulin genes used to prepare the phage library), it may be desireable to "backmutate" the altered framework residues of the selected antibodies to the germline configuration (i.e., change the framework amino acid sequences of the selected antibodies so that they are the same as the germline framework amino acid sequences). Such "backmutation" (or "germlining") of framework residues can be accomplished by standard molecular biology

methods for introducing specific mutations (e.g., site-directed mutagenesis; PCR-mediated mutagenesis, and the like).

Following screening and isolation of an anti-hTNFα antibody of the invention from a recombinant immunoglobulin display library, nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques. If desired, the nucleic acid can be further manipulated to create other antibody forms of the invention (e.g., linked to nucleic acid encoding additional immunoglobulin domains, such as additional constant regions). To express a recombinant human antibody isolated by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into a mammalian host cells, as described in further detail in Section II above.

IV. Pharmaceutical Compositions and Pharmaceutical Administration

The antibodies and antibody-portions of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody or antibody portion of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody or antibody portion.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies. The preferred mode of administration is parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i.e., antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The antibodies and antibody-portions of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, an antibody or antibody portion of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating disorders in which TNFα activity is detrimental. For example, an anti-hTNFα antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (e.g., antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see e.g., PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexanylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies.

Nonlimiting examples of therapeutic agents for rheumatoid arthritis with which an antibody, or antibody portion, of the invention can be combined include the following: nonsteroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/

BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffman-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see e.g., *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see e.g., *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNFR (soluble TNF binding protein; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol.-Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (e.g., Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see e.g., *Neuro Report* (1996) Vol. 7 pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see e.g., Arthritis & Rheumatism (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (e.g., SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; interleukin-11 (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see e.g., *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see e.g., DeLuca et al. (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); and azaribine.

Nonlimiting examples of therapeutic agents for inflammatory bowel disease with which an antibody, or antibody portion, of the invention can be combined include the following: budenoside; epidermal growth factor; corticosteroids; cyclosporin, sulfasalazine; aminosalicylates; 6-mercaptopurine; azathioprine; metronidazole; lipoxygenase inhibitors; mesalamine; olsalazine; balsalazide; antioxidants; thromboxane inhibitors; IL-1 receptor antagonists; anti-IL-1β monoclonal antibodies; anti-IL-6 monoclonal antibodies; growth factors; elastase inhibitors; pyridinyl-imidazole compounds; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); interleukin-10 (SCH 52000; Schering Plough); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); interleukin-11; glucuronide- or dextran-conjugated prodrugs of prednisolone, dexamethasone or budesonide; ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); slow-release mesalazine; methotrexate; antagonists of Platelet Activating Factor (PAF); ciprofloxacin; and lignocaine.

Nonlimiting examples of therapeutic agents for multiple sclerosis with which an antibody, or antibody portion, of the invention can be combined include the following: corticosteroids; prednisolone; methylprednisolone; azathioprine; cyclophosphamide; cyclosporine; methotrexate; 4-aminopyridine; tizanidine; interferon-β1a (Avonex™; Biogen); interferon-β1b (Betaseron™; Chiron/Berlex); Copolymer 1 (Cop-1; Copaxone™; Teva Pharmaceutical Industries, Inc.); hyperbaric oxygen; intravenous immunoglobulin; clabribine; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IL-10; IL-4; and IL-10 and/or IL-4 agonists (e.g., agonist antibodies).

Nonlimiting examples of therapeutic agents for sepsis with which an antibody, or antibody portion, of the invention can be combined include the following: hypertonic saline solutions; antibiotics; intravenous gamma globulin; continuous hemofiltration; carbapenems (e.g., meropenem); antagonists of cytokines such as TNFα, IL-1β, IL-6 and/or IL-8; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); SK&F 107647 (low molecular peptide; SmithKline Beecham); tetravalent

guanylhydrazone CNI-1493 (Picower Institute); Tissue Factor Pathway Inhibitor (TFPI; Chiron); PHP (chemically modified hemoglobin; APEX Bioscience); iron chelators and chelates, including diethylenetriamine pentaacetic acid—iron (III) complex (DTPA iron (III); Molichem Medicines); lisofylline (synthetic small molecule methylxanthine; Cell Therapeutics, Inc.); PGG-Glucan (aqeuous soluble β1,3glucan; Alpha-Beta Technology); apolipoprotein A-1 reconstituted with lipids; chiral hydroxamic acids (synthetic antibacterials that inhibit lipid A biosynthesis); anti-endotoxin antibodies; E5531 (synthetic lipid A antagonist; Eisai America, Inc.); $rBPI_{21}$ (recombinant N-terminal fragment of human Bactericidal/Permeability-Increasing Protein); and Synthetic Anti-Endotoxin Peptides (SAEP; BiosYnth Research Laboratories);

Nonlimiting examples of therapeutic agents for adult respiratory distress syndrome (ARDS) with which an antibody, or antibody portion, of the invention can be combined include the following: anti-IL-8 antibodies; surfactant replacement therapy; CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2 (chimeric anti-TNFα antibody; Centocor); 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); and 55 kdTNFR-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche).

The use of the antibodies, or antibody portions, of the invention in combination with other therapeutic agents is discussed further in subsection IV.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody or antibody portion may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 0.1-20 mg/kg, more preferably 1-10 mg/kg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

IV. Uses of the Antibodies of the Invention

Given their ability to bind to hTNFα, the anti-hTNFα antibodies, or portions thereof, of the invention can be used to detect hTNFα (e.g., in a biological sample, such as serum or plasma), using a conventional immunoassay, such as an enzyme linked immunosorbent assays (ELISA), an radioimmunoassay (RIA) or tissue immunohistochemistry. The invention provides a method for detecting hTNFα in a biological sample comprising contacting a biological sample with an antibody, or antibody portion, of the invention and detecting either the antibody (or antibody portion) bound to hTNFα or unbound antibody (or antibody portion), to thereby detect hTNFα in the biological sample. The antibody is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Alternative to labeling the antibody, hTNFα can be assayed in biological fluids by a competition immunoassay utilizing rhTNFα standards labeled with a detectable substance and an unlabeled anti-hTNFα antibody. In this assay, the biological sample, the labeled rhTNFα standards and the anti-hTNFα antibody are combined and the amount of labeled rhTNFα standard bound to the unlabeled antibody is determined. The amount of hTNFα in the biological sample is inversely proportional to the amount of labeled rhTNFα standard bound to the anti-hTNFα antibody.

A D2E7 antibody of the invention can also be used to detect TNFαs from species other than humans, in particular TNFαs from primates (e.g., chimpanzee, baboon, marmoset, cynomolgus and rhesus), pig and mouse, since D2E7 can bind to each of these TNFαs (discussed further in Example 4, subsection E).

The antibodies and antibody portions of the invention are capable of neutralizing hTNFα activity both in vitro and in vivo (see Example 4). Moreover, at least some of the antibodies of the invention, such as D2E7, can neutralize TNFα activity from other species. Accordingly, the antibodies and antibody portions of the invention can be used to inhibit TNFα activity, e.g., in a cell culture containing hTNFα, in human subjects or in other mammalian subjects having

TNFαs with which an antibody of the invention cross-reacts (e.g. chimpanzee, baboon, marmoset, cynomolgus and rhesus, pig or mouse). In one embodiment, the invention provides a method for inhibiting TNFα activity comprising contacting TNFα with an antibody or antibody portion of the invention such that TNFα activity is inhibited. Preferably, the TNFα is human TNFα. For example, in a cell culture containing, or suspected of containing hTNFα, an antibody or antibody portion of the invention can be added to the culture medium to inhibit hTNFα activity in the culture.

In another embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from a disorder in which TNFα activity is detrimental. TNFα has been implicated in the pathophysiology of a wide variety of disorders (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162-169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A.). The invention provides methods for TNFα activity in a subject suffering from such a disorder, which method comprises administering to the subject an antibody or antibody portion of the invention such that TNFα activity in the subject is inhibited. Preferably, the TNFα is human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (e.g., by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time courses of administration).

As used herein, the term "a disorder in which TNFα activity is detrimental" is intended to include diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which TNFα activity is detrimental is a disorder in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (e.g., an increase in the concentration of TNFα in serum, plasma, synovial fluid, etc. of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. There are numerous examples of disorders in which TNFα activity is detrimental. The use of the antibodies and antibody portions of the invention in the treatment of specific disorders is discussed further below:

A. Sepsis

Tumor necrosis factor has an established role in the pathophysiology of sepsis, with biological effects that include hypotension, myocardial suppression, vascular leakage syndrome, organ necrosis, stimulation of the release of toxic secondary mediators and activation of the clotting cascade (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162-169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A.; Tracey, K. J. and Cerami, A. (1994) *Annu. Rev. Med.* 45:491-503; Russell, D and Thompson, R. C. (1993) *Curr. Opin. Biotech.* 4:714-721). Accordingly, the human antibodies, and antibody portions, of the invention can be used to treat sepsis in any of its clinical settings, including septic shock, endotoxic shock, gram negative sepsis and toxic shock syndrome.

Furthermore, to treat sepsis, an anti-hTNFα antibody, or antibody portion, of the invention can be coadministered with one or more additional therapeutic agents that may further alleviate sepsis, such as an interleukin-1 inhibitor (such as those described in PCT Publication Nos. WO 92/16221 and WO 92/17583), the cytokine interleukin-6 (see e.g., PCT Publication No. WO 93/11793) or an antagonist of platelet activating factor (see e.g., European Patent Application Publication No. EP 374 510). Other combination therapies for the treatment of sepsis are discussed further in subsection III.

Additionally, in a preferred embodiment, an anti-TNFα antibody or antibody portion of the invention is administered to a human subject within a subgroup of sepsis patients having a serum or plasma concentration of L-6 above 500 pg/ml, and more preferably 1000 pg/ml, at the time of treatment (see PCT Publication No. WO 95/20978 by Daum, L., et al.).

B. Autoimmune Diseases

Tumor necrosis factor has been implicated in playing a role in the pathophysiology of a variety of autoimmune diseases. For example, TNFα has been implicated in activating tissue inflammation and causing joint destruction in rheumatoid arthritis (see e.g., Moeller, A., et al. (1990) *Cytokine* 2:162-169; U.S. Pat. No. 5,231,024 to Moeller et al.; European Patent Publication No. 260 610 B1 by Moeller, A.; Tracey and Cerami, supra; Arend, W. P. and Dayer, J- M. (1995) *Arth. Rheum.* 38:151-160; Fava, R. A., et al. (1993) *Clin. Exp. Immunol.* 94:261-266). TNFα also has been implicated in promoting the death of islet cells and in mediating insulin resistance in diabetes (see e.g., Tracey and Cerami, supra; PCT Publication No. WO 94/08609). TNFα also has been implicated in mediating cytotoxicity to oligodendrocytes and induction of inflammatory plaques in multiple sclerosis (see e.g., Tracey and Cerami, supra). Chimeric and humanized murine anti-hTNFα antibodies have undergone clinical testing for treatment of rheumatoid arthritis (see e.g., Elliott, M. J., et al. (1994) *Lancet* 344:1125-1127; Elliot, M. J., et al. (1994) *Lancet* 344:1105-1110; Rankin, E. C., et al. (1995) *Br. J Rheumatol.* 34:334-342).

The human antibodies, and antibody portions of the invention can be used to treat autoimmune diseases, in particular those associated with inflammation, including rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis and gouty arthritis, allergy, multiple sclerosis, autoimmune diabetes, autoimmune uveitis and nephrotic syndrome. Typically, the antibody, or antibody portion, is administered systemically, although for certain disorders, local administration of the antibody or antibody portion at a site of inflammation may be beneficial (e.g., local administration in the joints in rheumatoid arthritis or topical application to diabetic ulcers, alone or in combination with a cyclohexane-ylidene derivative as described in PCT Publication No. WO 93/19751). An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of autoimmune diseases, as discussed further in subsection III.

C. Infectious Diseases

Tumor necrosis factor has been implicated in mediating biological effects observed in a variety of infectious diseases. For example, TNFα has been implicated in mediating brain inflammation and capillary thrombosis and infarction in malaria. TNFα also has been implicated in mediating brain inflammation, inducing breakdown of the blood-brain barrier, triggering septic shock syndrome and activating venous inf-

arction in meningitis. TNFα also has been implicated in inducing cachexia, stimulating viral proliferation and mediating central nervous system injury in acquired immune deficiency syndrome (AIDS). Accordingly, the antibodies, and antibody portions, of the invention, can be used in the treatment of infectious diseases, including bacterial meningitis (see e.g., European Patent Application Publication No. EP 585 705), cerebral malaria, AIDS and AIDS-related complex (ARC) (see e.g., European Patent Application Publication No. EP 230 574), as well as cytomegalovirus infection secondary to transplantation (see e.g., Fietze, E., et al. (1994) *Transplantation* 58:675-680). The antibodies, and antibody portions, of the invention, also can be used to alleviate symptoms associated with infectious diseases, including fever and myalgias due to infection (such as influenza) and cachexia secondary to infection (e.g., secondary to AIDS or ARC).

D. Transplantation

Tumor necrosis factor has been implicated as a key mediator of allograft rejection and graft versus host disease (GVHD) and in mediating an adverse reaction that has been observed when the rat antibody OKT3, directed against the T cell receptor CD3 complex, is used to inhibit rejection of renal transplants (see e.g., Eason, J. D., et al. (1995) *Transplantation* 59:300-305; Suthanthiran, M. and Strom, T. B. (1994) *New Engl. J Med.* 331:365-375). Accordingly, the antibodies, and antibody portions, of the invention, can be used to inhibit transplant rejection, including rejections of allografts and xenografts and to inhibit GVHD. Although the antibody or antibody portion may be used alone, more preferably it is used in combination with one or more other agents that inhibit the immune response against the allograft or inhibit GVHD. For example, in one embodiment, an antibody or antibody portion of the invention is used in combination with OKT3 to inhibit OKT3-induced reactions. In another embodiment, an antibody or antibody portion of the invention is used in combination with one or more antibodies directed at other targets involved in regulating immune responses, such as the cell surface molecules CD25 (interleukin-2 receptor-α), CD11a (LFA-1), CD54 (ICAM- 1), CD4, CD45, CD28/CTLA4, CD80 (B7-1) and/or CD86 (B7-2). In yet another embodiment, an antibody or antibody portion of the invention is used in combination with one or more general immunosuppressive agents, such as cyclosporin A or FK506.

E. Malignancy

Tumor necrosis factor has been implicated in inducing cachexia, stimulating tumor growth, enhancing metastatic potential and mediating cytotoxicity in malignancies. Accordingly, the antibodies, and antibody portions, of the invention, can be used in the treatment of malignancies, to inhibit tumor growth or metastasis and/or to alleviate cachexia secondary to malignancy. The antibody, or antibody portion, may be administered systemically or locally to the tumor site.

F. Pulmonary Disorders

Tumor necrosis factor has been implicated in the pathophysiology of adult respiratory distress syndrome (ARDS), including stimulating leukocyte-endothelial activation, directing cytotoxicity to pneumocytes and inducing vascular leakage syndrome. Accordingly, the antibodies, and antibody portions, of the invention, can be used to treat various pulmonary disorders, including adult respiratory distress syndrome (see e.g., PCT Publication No. WO 91/04054), shock lung, chronic pulmonary inflammatory disease, pulmonary sarcoidosis, pulmonary fibrosis and silicosis. The antibody, or antibody portion, may be administered systemically or locally to the lung surface, for example as an aerosol. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of pulmonary disorders, as discussed further in subsection III.

G. Intestinal Disorders

Tumor necrosis factor has been implicated in the pathophysiology of inflammatory bowel disorders (see e.g., Tracy, K. J., et al. (1986) *Science* 234:470-474; Sun, X- M., et al. (1988) *J. Clin. Invest.* 81:1328-1331; MacDonald, T. T., et al. (1990) *Clin. Exp. Immunol.* 81:301-305). Chimeric murine anti-hTNFα antibodies have undergone clinical testing for treatment of Crohn's disease (van Dullemen, H. M., et al. (1995) *Gastroenterology* 109:129-135). The human antibodies, and antibody portions, of the invention, also can be used to treat intestinal disorders, such as idiopathic inflammatory bowel disease, which includes two syndromes, Crohn's disease and ulcerative colitis. An antibody, or antibody portion, of the invention also can be administered with one or more additional therapeutic agents useful in the treatment of intestinal disorders, as discussed further in subsection III.

H. Cardiac Disorders

The antibodies, and antibody portions, of the invention, also can be used to treat various cardiac disorders, including ischemia of the heart (see e.g., European Patent Application Publication No. EP 453 898) and heart insufficiency (weakness of the heart muscle)(see e.g., PCT Publication No. WO 94/20139).

I. Others

The antibodies, and antibody portions, of the invention, also can be used to treat various other disorders in which TNFα activity is detrimental. Examples of other diseases and disorders in which TNFα activity has been implicated in the pathophysiology, and thus which can be treated using an antibody, or antibody portion, of the invention, include inflammatory bone disorders and bone resorption disease (see e.g., Bertolini, D. R., et al. (1986) *Nature* 319:516-518; Konig, A., et al. (1988) *J. Bone Miner. Res.* 3:621-627; Lemer, U. H. and Ohlin, A. (1993) *J. Bone Miner. Res.* 8:147-155; and Shankar, G. and Stern, P. H. (1993) *Bone* 14:871-876), hepatitis, including alcoholic hepatitis (see e.g., McClain, C. J. and Cohen, D. A. (1989) *Hepatology* 9:349-351; Felver, M. E., et al. (1990) *Alcohol. Clin. Exp. Res.* 14:255-259; and Hansen, J., et al. (1994) *Hepatology* 20:461-474), viral hepatitis (Sheron, N., et al. (1991) *J. Hepatol.* 12:241-245; and Hussain, M. J., et al. (1994) *J. Clin. Pathol.* 47:1112-1115), and fulminant hepatitis; coagulation disturbances (see e.g., van der Poll, T., et al. (1990) *N. Engl. J Med.* 322:1622-1627; and van der Poll, T., et al. (1991) *Prog. Clin. Biol. Res.* 367:55-60), burns (see e.g., Giroir, B. P., et al. (1994) *Am. J. Physiol.* 267:H118-124; and Liu, X. S., et al. (1994) *Burns* 20:40-44), reperfusion injury (see e.g., Scales, W. E., et al. (1994) *Am. J. Physiol.* 267:G1 122-1127; Serrick, C., et al. (1994) *Transplantation* 58:1158-1162; and Yao, Y. M., et al. (1995) *Resuscitation* 29:157-168), keloid formation (see e.g., McCauley, R. L., et al. (1992) *J. Clin. Immunol.* 12:300-308), scar tissue formation; pyrexia; periodontal disease; obesity and radiation toxicity.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLE 1

Kinetic Analysis of Binding of Human Antibodies to hTNFα

Real-time binding interactions between ligand (biotinylated recombinant human TNFα (rhTNFα) immobilized on a biosensor matrix) and anialyte (antibodies in solution) were measured by surface plasmon resonance (SPR) using the BIAcore system (Pharmacia Biosensor, Piscataway, N.J.). The system utilizes the optical properties of SPR to detect alterations in protein concentrations within a dextran biosensor matrix. Proteins are covalently bound to the dextran matrix at known concentrations. Antibodies are injected through the dextran matrix and specific binding between injected antibodies and immobilized ligand results in an increased matrix protein concentration and resultant change in the SPR signal. These changes in SPR signal are recorded as resonance units (RU) and are displayed with respect to time along the y-axis of a sensorgram.

To facilitate immobilization of biotinylated rhTNFα on the biosensor matrix, streptavidin is covalently linked via free amine groups to the dextran matrix by first activating carboxyl groups on the matrix with 100 mM N-hydroxysuccinimide (NHS) and 400 mM N-ethyl-N'-(3-diethylaminopropyl) carbodimide hydrochloride (EDC). Next, streptavidin is injected across the activated matrix. Thirty-five microliters of streptavidin (25 μg/ml), diluted in sodium acetate, pH 4.5, is injected across the activated biosensor and free amines on the protein are bound directly to the activated carboxyl groups. Unreacted matrix EDC-esters are deactivated by an injection of 1 M ethanolamine. Streptavidin-coupled biosensor chips also are commercially available (Pharmacia BR-1000-16, Pharmacia Biosensor, Piscataway, N.J.).

Biotinylated rhTNFα was prepared by first dissolving 5.0 mg of biotin (D-biotinyl-ε-aminocaproic acid N-hydroxysuccinimide ester; Boehringer Mannheim Cat. No. 1008 960) in 500 μl dimethylsulfoxide to make a 10 mg/ml solution. Ten microliters of biotin was added per ml of rhTNFα (at 2.65 mg/ml) for a 2:1 molar ratio of biotin to rhTNFα. The reaction was mixed gently and incubated for two hours at room temperature in the dark. A PD-10 column, Sephadex G-25M (Pharmacia Catalog No. 17-0851-01) was equilibrated with 25 ml of cold PBS and loaded with 2 ml of rhTNFα-biotin per column. The column was eluted with 10×1 ml cold PBS. Fractions were collected and read at OD280 (1.0 OD=1.25 mg/ml). The appropriate fractions were pooled and stored at −80° C. until use. Biotinylated rhTNFα also is commercially available (R & D Systems Catalog No. FTA00, Minneapolis, Minn.).

Biotinylated rhTNFα to be immobilized on the matrix via streptavidin was diluted in PBS running buffer (Gibco Cat. No. 14190-144, Gibco BRL, Grand Island, N.Y.) supplemented with 0.05% (BIAcore) surfactant P20 (Pharmacia BR-1000-54, Pharmacia Biosensor, Piscataway, N.J.). To determine the capacity of rhTNFα-specific antibodies to bind immobilized rhTNFα, a binding assay was conducted as follows. Aliquots of biotinylated rhTNFα (25 nM; 10 μl aliquots) were injected through the streptavidin-coupled dextran matrix at a flow rate of 5 μl/min. Before injection of the protein and immediately afterward, PBS buffer alone flowed through each flow cell. The net difference in signal between baseline and approximately 30 sec. after completion of biotinylated rhTNFα injection was taken to represent the binding value (approximately 500 RU). Direct rhTNFα-specific antibody binding to immobilized biotinylated rhTNFα was measured. Antibodies (20 μg/ml) were diluted in PBS running buffer and 25 μl aliquots were injected through the immobilized protein matrices at a flow rate of 5 μl/min. Prior to injection of antibody, and immediately afterwards, PBS buffer alone flowed through each flow cell. The net difference in baseline signal and signal after completion of antibody injection was taken to represent the binding value of the particular sample. Biosensor matrices were regenerated using 100 mM HCl before injection of the next sample. To determine the off rate (K_{off}), on rate (K_{on}), association rate (K_a) and dissociation rate (K_d) constants, BIAcore kinetic evaluation software (version 2.1) was used.

Representative results of D2E7 (IgG4 full-length antibody) binding to biotinylated rhTNFα, as compared to the mouse mAb MAK 195 ($F(ab')_2$ fragment), are shown below in Table 1.

TABLE 1

Binding of D2E7 IgG4 or MAK 195 to Biotinylated rhTNFα

Antibody	[Ab], nM	rhTNFα, bound, RUs	Ab, bound, RUs	rhTNFα/Ab	K_{off}, sec^{-1}, (Avg)
D2E7	267	373	1215	1.14	8.45×10^{-5}
	133	420	1569	1.30	5.42×10^{-5}
	67	434	1633	1.31	4.75×10^{-5}
	33	450	1532	1.19	4.46×10^{-5}
	17	460	1296	0.98	3.47×10^{-5}
	8	486	936	0.67	2.63×10^{-5}
	4	489	536	0.38	2.17×10^{-5}
	2	470	244	0.18	3.68×10^{-5}
					(4.38×10^{-5})
MAK 195	400	375	881	1.20	5.38×10^{-5}
	200	400	1080	1.38	4.54×10^{-5}
	100	419	1141	1.39	3.54×10^{-5}
	50	427	1106	1.32	3.67×10^{-5}
	25	446	957	1.09	4.41×10^{-5}
	13	464	708	0.78	3.66×10^{-5}
	6	474	433	0.47	7.37×10^{-5}
	3	451	231	0.26	6.95×10^{-5}
					(4.94×10^{-5})

In a second series of experiments, the molecular kinetic interactions between an IgG1 full-length form of D2E7 and biotinylated rhTNF was quantitatively analyzed using BIAcore technology, as described above, and kinetic rate constants were derived, summarized below in Tables 2, 3 and 4.

TABLE 2

Apparent dissociation rate constants of the interaction between D2E7 and biotinylated rhTNF

Experiment	K_d (s^{-1})
1	9.58×10^{-5}
2	9.26×10^{-5}
3	7.60×10^{-5}
Average	$8.81 \pm 1.06 \times 10^{-5}$

TABLE 3

Apparent association rate constants of the interaction between D2E7 and biotinylated rhTNF

Experiment	K_a (M^{-1}, s^{-1})
1	1.33×10^5
2	1.05×10^5
3	3.36×10^5
Average	$1.91 \pm 1.26 \times 10^5$

TABLE 4

Apparent kinetic reate and affinity constants of D2E7 and biotinylated rhTNF

Experiment	K_a (M^{-1}, s^{-1})	K_d (s^{-1})	K_d (M)
1	1.33×10^5	9.58×10^{-5}	7.20×10^{-10}
2	1.05×10^5	9.26×10^{-5}	8.82×10^{-10}
3	3.36×10^5	7.60×10^{-5}	2.26×10^{-10}
Average	$1.91 \pm 1.26 \times 10^5$	$8.81 \pm 1.06 \times 10^{-5}$	$6.09 \pm 3.42 \times 10^{-10}$

Dissociation and association rate constants were calculated by analyzing the dissociation and association regions of the sensorgrams by BIA analysis software. Conventional chemical reaction kinetics were assumed for the interaction between D2E7 and biotinylated rhTNF molecule: a zero order dissociation and first order association kinetics. For the sake of analysis, interaction only between one arm of the bivalent D2E7 antibody and one unit of the trimeric biotinylated rhTNF was considered in choosing molecular models for the analysis of the kinetic data. Three independent experiments were performed and the results were analyzed separately. The average apparent dissociation rate constant (k_d) of the interaction between D2E7 and biotinylated rhTNF was $8.81 \pm 1.06 \times 10^{-5}$ s^{-1}, and the average apparent association rate constant, k_a was $1.91 \pm 1.26 \times 10^5$ M^{-1} s^{-1}. The apparent intrinsic dissociation constant (K_d) was then calculated by the formula: $K_d = k_d/k_a$. Thus, the mean K_d of D2E7 antibody for rhTNF derived from kinetic parameters was $6.09 \pm 3.42 \times 10^{-10}$ M. Minor differences in the kinetic values for the IgG1 form of D2E7 (presented in Tables 2, 3 and 4) and the IgG4 form of D2E7 (presented in Table 1 and in Examples 2 and 3) are not thought to be true differences resulting from the presence of either an IgG1 or an IgG4 constant regions but rather are thought to be attributable to more accurate antibody concentration measurements used for the IgG1 kinetic analysis. Accordingly, the kinetic values for the IgG1 form of D2E7 presented herein are thought to be the most accurate kinetic parameters for the D2E7 antibody.

EXAMPLE 2

Alanine Scanning Mutagenesis of D2E7 CDR3 Domains

A series of single alanine mutations were introduced by standard methods along the CDR3 domain of the D2E7 VL and the D2E7 VH regions. The light chain mutations are illustrated in FIG. 1B (LD2E7*.A1, LD2E7*.A3, LD2E7*.A4, LD2E7*.A5, LD2E7*.A7 and LD2E7*.A8, having an alanine mutation at position 1, 3, 4, 5, 7 or 8, respectively, of the D2E7 VL CDR3 domain). The heavy chain mutations are illustrated in FIG. 2B (HD2E7*.A1, HD2E7*.A2, HD2E7*.A3, HD2E7*.A4, HD2E7*.A5, HD2E7*.A6, HD2E7*.A7, HD2E7*.A8 and HD2E7*.A9, having an alanine mutation at position 2, 3, 4, 5, 6, 8, 9, 10 or 11, respectively, of the D2E7 VH CDR3 domain). The kinetics of rhTNFα interaction with an antibody composed of wild-type D2E7 VL and VH was compared to that of antibodies composed of 1) a wild-type D2E7 VL paired with an alanine-substituted D2E7 VH; 2) a wild-type D2E7 VH paired with an alanine-substituted D2E7 VL; or 3) an alanine-substituted D2E7 VL paired with an alanine-substituted D2E7 VH. All antibodies were tested as full-length, IgG4 molecules.

Kinetics of interaction of antibodies with rhTNFα was determined by surface plasmon resonance as described in Example 1. The K_{off} rates for the different VH/VL pairs are summarized below in Table 5:

TABLE 5

Binding of D2E7 Alanine-Scan Mutants to Biotinylated rhTNFα

VH	VL	K_{off} (sec^{-1})
D2E7 VH	D2E7 VL	9.65×10^{-5}
HD2E7*.A1	D2E7 VL	1.4×10^{-4}
HD2E7*.A2	D2E7 VL	4.6×10^{-4}
HD2E7*.A3	D2E7 VL	8.15×10^{-4}
HD2E7*.A4	D2E7 VL	1.8×10^{-4}
HD2E7*.A5	D2E7 VL	2.35×10^{-4}
HD2E7*.A6	D2E7 VL	2.9×10^{-4}
HD2E7*.A7	D2E7 VL	1.0×10^{-4}
HD2E7*.A8	D2E7 VL	3.1×10^{-4}
HD2E7*.A9	D2E7 VL	8.1×10^{-4}
D2E7 VH	LD2E7*.A1	6.6×10^{-5}
D2E7 VH	LD2E7*.A3	NOT DETECTABLE
D2E7 VH	LD2E7*.A4	1.75×10^{-4}
D2E7 VH	LD2E7*.A5	1.8×10^{-4}
D2E7 VH	LD2E7*.A7	1.4×10^{-4}
D2E7 VH	LD2E7*.A8	3.65×10^{-4}
HD2E7*.A9	LD2E7*.A1	1.05×10^{-4}

These results demonstrate that the majority of positions of the CDR3 domains of the D2E7 VL region and VH region are amenable to substitution with a single alanine residue. Substitution of a single alanine at position 1, 4, 5, or 7 of the D2E7 VL CDR3 domain or at position 2, 5, 6, 8, 9 or 10 of the D2E7 VH CDR3 domain does not significantly affect the off rate of hTNFα binding as compared to the wild-type parental D2E7 antibody. Substitution of alanine at position 8 of the D2E7 VL CDR3 or at position 3 of the D2E7 VH CDR3 gives a 4-fold faster K_{off} and an alanine substitution at position 4 or 11 of D2E7 VH CDR3 gives an 8-fold faster K_{off}, indicating that these positions are more critical for binding to hTNFα. However, a single alanine substitution at position 1, 4, 5, 7 or 8 of the D2E7 VL CDR3 domain or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 of the D2E7 VH CDR3 domain still results in an anti-hTNFα antibody having a K_{off} of 1×10^{-3} sec-1 or less.

EXAMPLE 3

Binding Analysis of D2E7-Related Antibodies

A series of antibodies related in sequence to D2E7 were analyzed for their binding to rhTNFα, as compared to D2E7, by surface plasmon resonance as described in Example 1. The amino acid sequences of the VL regions tested are shown in FIGS. 1A and 1B. The amino acid sequences of the VH regions tested are shown in FIGS. 2A and 2B. The K_{off} rates for various VH/VL pairs (in the indicated format, either as a full-length IgG1 or IgG4 antibody or as a scFv) are summarized below in Table 6:

TABLE 6

Binding of D2E7-Related Antibodies to Biotinylated rhTNFα

VH	VL	Format	K_{off} (sec^{-1})
D2E7 VH	D2E7 VL	IgG1/IgG4	9.65×10^{-5}
VH1-D2	LOE7	IgG1/IgG4	7.7×10^{-5}
VH1-D2	LOE7	scFv	4.6×10^{-4}
VH1-D2.N	LOE7.T	IgG4	2.1×10^{-5}
VH1-D2.Y	LOE7.A	IgG4	2.7×10^{-5}
VH1-D2.N	LOE7.A	IgG4	3.2×10^{-5}

TABLE 6-continued

Binding of D2E7-Related Antibodies to Biotinylated rhTNFα

VH	VL	Format	K_{off} (sec^{-1})
VH1-D2	EP B12	scFv	8.0×10^{-4}
VH1-D2	2SD4 VL	scFv	1.94×10^{-3}
3C-H2	LOE7	scFv	1.5×10^{-3}
2SD4 VH	LOE7	scFv	6.07×10^{-3}
2SD4 VH	2SD4 VL	scFv	1.37×10^{-2}
VH1A11	2SD4 VL	scFv	1.34×10^{-2}
VH1B12	2SD4 VL	scFv	1.01×10^{-2}
VH1B11	2SD4 VL	scFv	9.8×10^{-3}
VH1E4	2SD4 VL	scFv	1.59×10^{-2}
VH1F6	2SD4 VL	scFv	2.29×10^{-2}
VH1D8	2SD4 VL	scFv	9.5×10^{-3}
VH1G1	2SD4 VL	scFv	2.14×10^{-2}
2SD4 VH	EP B12	scFv	6.7×10^{-3}
2SD4 VH	VL10E4	scFv	9.6×10^{-3}
2SD4 VH	VL100A9	scFv	1.33×10^{-2}
2SD4 VH	VL100D2	scFv	1.41×10^{-2}
2SD4 VH	VL10F4	scFv	1.11×10^{-2}
2SD4 VH	VLLOE5	scFv	1.16×10^{-2}
2SD4 VH	VLL0F9	scFv	6.09×10^{-3}
2SD4 VH	VLL0F10	scFv	1.34×10^{-2}
2SD4 VH	VLLOG7	scFv	1.56×10^{-2}
2SD4 VH	VLLOG9	scFv	1.46×10^{-2}
2SD4 VH	VLLOH1	scFv	1.17×10^{-2}
2SD4 VH	VLLOH10	scFv	1.12×10^{-2}
2SD4 VH	VL1B7	scFv	1.3×10^{-2}
2SD4 VH	VL1C1	scFv	1.36×10^{-2}
2SD4 VH	VL1C7	scFv	2.0×10^{-2}
2SD4 VH	VL0.1F4	scFv	1.76×10^{-2}
2SD4 VH	VL0.1H8	scFv	1.14×10^{-2}

The slow off rates (i.e., $K_{off} \leqq 1 \times 10^{-4}$ sec^{-1}) for full-length antibodies (i.e., IgG format) having a VL selected from D2E7, LOE7, LOE7.T and LOE7.A, which have either a threonine or an alanine at position 9, indicate that position 9 of the D2E7 VL CDR3 can be occupied by either of these two residues without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R—Y—N—R-A-P—Y-(T/A) (SEQ ID NO: 3). Furthermore, the slow off rates (i.e., $K_{off} \leqq 1 \times 10^{-4}$ sec^{-1}) for antibodies having a VH selected from D2E7, VH1-D2.N and VH1 -D2.Y, which have either a tyrosine or an asparagine at position 12, indicate that position 12 of the D2E7 VH CDR3 can be occupied by either of these two residues without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V—S—Y-L-S—T-A-S—S-L-D-(Y/N) (SEQ ID NO: 4).

The results shown in Table 6 demonstrate that, in scFv format, antibodies containing the 2SD4 VL or VH CDR3 region exhibit a faster K_{off} (i.e., $K_{off} \geqq 1 \times 10^{-3}$ sec^{-1}) as compared to antibodies containing the D2E7 VL or VH CDR3 region. Within the VL CDR3, 2SD4 differs from D2E7 at positions 2, 5 and 9. As discussed above, however, position 9 may be occupied by Ala (as in 2SD4) or Thr (as in D2E7) without substantially affecting the K_{off}. Thus, by comparison of 2SD4 and D2E7, positions 2 and 5 of the D2E7 VL CDR3, both arginines, can be identified as being critical for the association of the antibody with hTNFα. These residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Regarding the importance of position 2, replacement of Arg (in LOE7, which has the same VL CDR3 as D2E7) with Lys (in EP B12) accelerates the off rate by a factor of two. Regarding the importance of position 5, replacement of Arg (in D2E7) with Ala (in LD2E7*.A5), as described in Example 2, also accelerates the off rate two-fold. Furthermore, without either Arg at positions 2 and 5 (in 2SD4), the off rate is five-fold faster. However, it should be noted that although position 5 is important for improved binding to hTNFα, a change at this position can be negated by changes at other positions, as seen in VLLOE4, VLLOH1 or VL0.1H8.

Within the VH CDR3, 2SD4 differs from D2E7 at positions 1, 7 and 12. As discussed above, however, position 12 may be occupied by Asn (as in 2SD4) or Tyr (as in D2E7) without substantially affecting the K_{off}. Thus, by comparison of 2SD4 and D2E7, positions 1 and 7 of the D2E7 VH CDR3 can be identified as being critical for binding to hTNFα. As discussed above, these residues could be directly involved as contact residues in the antibody binding site or could contribute critically to maintaining the scaffolding architecture of the antibody molecule in this region. Both positions are important for binding to hTNFα since when the 3C H2 VH CDR3 (which has a valine to alanine change at position 1 with respect to the D2E7 VH CDR3) is used, the scFv has a 3-fold faster off rate than when the D2E7 VH CDR3 is used but this off rate is still four times slower than when the 2SD4 VH CDR3 is used (which has changes at both positions 1 and 7 with respect to the D2E7 VH CDR3).

EXAMPLE 4

Functional Activity of D2E7

To examine the functional activity of D2E7, the antibody was used in several assays that measure the ability of the antibody to inhibit hTNFα activity, either in vitro or in vivo.

A. Neutralization of TNFα-Induced Cytotoxicity in L929 Cells

Human recombinant TNFα (rhTNFα) causes cell cytotoxicity to murine L929 cells after an incubation period of 18-24 hours. Human anti-hTNFα antibodies were evaluated in L929 assays by coincubation of antibodies with rhTNFα and the cells as follows. A 96-well microtiter plate containing 100 μl of anti-hTNFα Abs was serially diluted ½ down the plate in duplicates using RPMI medium containing 10% fetal bovine serum (FBS). Fifty microliters of rhTNFα was added for a final concentration of 500 pg/ml in each sample well. The plates were then incubated for 30 minutes at room temperature. Next, 50 μl of TNFα-sensitive L929 mouse fibroblasts cells were added for a final concentration of 5×10^4 cells per well, including 1 μg/ml Actinomycin-D. Controls included medium plus cells and rhTNFα plus cells. These controls, and a TNFα standard curve, ranging from 2 ng/ml to 8.2 pg/ml, were used to determine the quality of the assay and provide a window of neutralization. The plates were then incubated overnight (18-24 hours) at 37° C. in 5% CO_2.

One hundred microliters of medium was removed from each well and 50 μl of 5 mg/ml 3,(4,4-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT; commercially available from Sigma Chemical Co., St. Louis, Mo.) in PBS was added. The plates were then incubated for 4 hours at 37° C. Fifty microliters of 20% sodium dodecyl sulfate (SDS) was then added to each well and the plates were incubated overnight at 37° C. The optical density at 570/630 nm was measured, curves were plotted for each sample and IC_{50}s were determined by standard methods.

Representative results for human antibodies having various VL and VH pairs, as compared to the murine MAK 195 mAb, are shown in FIG. 3 and in Table 7 below.

TABLE 7

Neutralization of TNFα-Induced L929 Cytotoxicity

VH	VL	Structure	IC_{50}, M
D2E7	D2E7	scFv	1.1×10^{-10}
D2E7	D2E7	IgG4	4.7×10^{-11}
2SD4	2SD4	scFv/IgG1/IgG4	3.0×10^{-7}
2SD4	LOE7	scFv	4.3×10^{-8}
VH1-D2	2SD4	scFv	1.0×10^{-8}
VH1-D2	LOE7	scFv/IgG1/IgG4	3.4×10^{-10}
VH1.D2.Y	LOE7.T	IgG4	8.1×10^{-11}
VH1-D2.N	LOE7.T	IgG4	1.3×10^{-10}
VH1-D2.Y	LOE7.A	IgG4	2.8×10^{-11}
VH1-D2.N	LOE7.A	IgG4	6.2×10^{-11}
MAK 195	MAK 195	scFv	1.9×10^{-8}
MAK 195	MAK195	$F(ab')_2$	6.2×10^{-11}

The results in FIG. 3 and Table 7 demonstrate that the D2E7 human anti-hTNFα antibody, and various D2E7-related antibodies, neutralize TNFα-induced L929 cytotoxicity with a capacity approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to neutralize TNFα-induced L929 cytotoxicity was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 8:

TABLE 8

Neutralization of TNFα-Induced L929 Cytotoxicity by D2E7 IgG1

Experiment	IC_{50} [M]
1	1.26×10^{-10}
2	1.33×10^{-10}
3	1.15×10^{-10}
Average	$1.25 \pm 0.01 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, neutralizes TNFα-induced L929 cytotoxicity with an average IC_{50} [M] of $1.25 \pm 0.01 \times 10^{-10}$.

B. Inhibition of TNFα Binding to TNFα Receptors on U-937 Cells

The ability of human anti-hTNFα antibodies to inhibit the binding of hTNFα to hTNFα receptors on the surface of cells was examined using the U-937 cell line (ATCC No. CRL 1593), a human histiocytic cell line that expresses hTNFα receptors. U-937 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone A-1111, Hyclone Laboratories, Logan, Utah), L-glutamine (4 nM), HEPES buffer solution (10 mM), penicillin (100 μg/ml) and streptomycin (100 μg/ml). To examine the activity of full-length IgG antibodies, U-937 cells were preincubated with PBS supplemented with 1 mg/ml of human IgG (Sigma I-4506, Sigma Chemical Co., St. Louis, Mo.) for 45 minutes on ice and then cells were washed three times with binding buffer. For the receptor binding assay, U-937 cells (5×10^6 cells/well) were incubated in a binding buffer (PBS supplemented with 0.2% bovine serum albumin) in 96-well microtiter plates (Costar 3799, Costar Corp., Cambridge, Mass.) together with ^{125}I-labeled rhTNFα (3×10^{-10} M; 25 μCi/ml; obtained from NEN Research Products, Wilmington, Del.), with or without anti-hTNFα antibodies, in a total volume of 0.2 ml. The plates were incubated on ice for 1.5 hours. Then, 75 μl of each sample was transferred to 1.0 ml test tubes (Sarstedt 72.700, Sarstedt Corp., Princeton, N.J.) containing dibutylphthalate (Sigma D-2270, Sigma Chemical Co., St. Louis, Mo.) and dinonylphthalate (ICN 210733, ICN, Irvine, Calif.). The test tubes contained a 300 μl mixture of dibutylphthalate and dinonylphthalate, 2:1 volume ratio, respectively. Free (i.e., unbound) ^{125}I-labeled rhTNFα was removed by microcentrifugation for five minutes. Then, each test tube end containing a cell pellet was cut with the aid of a microtube scissor (Bel-Art 210180001, Bel-Art Products, Pequannock, N.J.). The cell pellet contains ^{125}I-labeled rhTNFα bound to the p60 or p80 TNFα receptor, whereas the aqueous phase above the oil mixture contains excess free ^{125}I-labeled rhTNFα. All cell pellets were collected in a counting tube (Falcon 2052, Becton Dickinson Labware, Lincoln Park, N.J.) and counted in a scintillation counter.

Representative results are shown in FIG. 4. The IC_{50} value for D2E7 inhibition of hTNFα binding to hTNFα receptors on U-937 cells is approximately 3×10^{-10} M in these experiments. These results demonstrate that the D2E7 human anti-hTNFα antibody inhibits rhTNFα binding to hTNFα receptors on U-937 cells at concentrations approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit rhTNFα binding to hTNFα receptors on U-937 cells was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 9:

TABLE 9

Inhibition of TNF Receptor Binding on U-937 Cells by D2E7 IgG1

Experiment	IC_{50} [M]
1	1.70×10^{-10}
2	1.49×10^{-10}
3	1.50×10^{-10}
Average	$1.56 \pm 0.12 \times 10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNF receptor binding on U-937 cells with an average IC_{50} [M] of $1.56 \pm 0.12 \times 10^{-10}$.

To investigate the inhibitory potency of D2E7 in the binding of ^{125}I-rhTNF binding to individual p55 and p75 receptors, a solid phase radioimmunoassay was performed. To measure the IC_{50} values of D2E7 for separate TNF receptors, varying concentrations of the antibody were incubated with 3×10^{-10} concentration of 125I-rhTNF. The mixture was then tested on separate plates containing either the p55 or the p75 TNF receptors in a dose dependent manner. The results are summarized below in Table 10:

TABLE 10

Inhibition of TNF Receptor Binding to p55 and p75 TNFR by D2E7 IgG1

	IC_{50} [M]	
Reagent	p55 TNFR	p 75TNFR
D2E7	1.47×10^{-9}	1.26×10^{-9}
rhTNF	2.31×10^{-9}	2.70×10^{-9}

Inhibition of ^{125}I-rhTNF binding to the p55 and p75 TNF receptors on U937 cells by D2E7 followed a simple sigmoidal curve, indicating similar IC_{50} values for each receptor. In the solid phase radioimmunoassay (RIA) experiments with recombinant TNF receptors, IC_{50} values for inhibition of 125I-rhTNF binding to the p55 and the p75 receptors by

D2E7 were calculated as 1.47×10^{-9} and 1.26×10^{-9} M, respectively. The decrease in IC_{50} values in the solid phase was probably due to higher density of receptors in the RIA format, as unlabeled rhTNF also inhibited with similar IC_{50} values. The IC_{50} values for inhibition of ^{125}I-rhTNF binding to the p55 and the p75 receptors by unlabeled rhTNF were 2.31×10^{-9} and 2.70×10^{-9} M, respectively

C. Inhibition of ELAM-1 Expression on HUVEC

Human umbilical vein endothelial cells (HUVEC) can be induced to express endothelial cell leukocyte adhesion molecule 1 (ELAM-1) on their cell-surface by treatment with rhTNFα, which can be detected by reacting rhTNFα-treated HUVEC with an mouse anti-human ELAM-1 antibody. The ability of human anti-hTNFα antibodies to inhibit this TNFα-induced expression of ELAM-1 on HUVEC was examined as follows: HUVEC (ATCC No. CRL 1730) were plated in 96-well plates (5×10^4 cells/well) and incubated overnight at 37° C. The following day, serial dilutions of human anti-hTNFα antibody (1:10) were prepared in a microtiter plate, starting with 20-100 μg/ml of antibody. A stock solution of rhTNFα was prepared at 4.5 ng/ml, aliquots of rhTNFα were added to each antibody-containing well and the contents were mixed well. Controls included medium alone, medium plus anti-hTNFα antibody and medium plus rhTNFα. The HUVEC plates were removed from their overnight incubation at 37° C. and the medium gently aspirated from each well. Two hundred microliters of the antibody-rhTNFα mixture were transferred to each well of the HUVEC plates. The HUVEC plates were then further incubated at 37° C. for 4 hours. Next, a murine anti-ELAM-1 antibody stock was diluted 1:1000 in RPMI. The medium in each well of the HUJVEC plate was gently aspirated, 50 μl/well of the anti-ELAM-1 antibody solution was added and the HUVEC plates were incubated 60 minutes at room temperature. An ^{125}I-labeled anti-mouse Ig antibody solution was prepared in RPMI (approximately 50,000 cpm in 50 μl). The medium in each well of the HUVEC plates was gently aspirated, the wells were washed twice with RPMI and 50 μl of the ^{125}I-labeled anti-mouse Ig solution was added to each well. The plates were incubated for one hour at room temperature and then each well was washed three times with RPMI. One hundred eighty microliters of 5% SDS was added to each well to lyse the cells. The cell lysate from each well was then transferred to a tube and counted in a scintillation counter.

Representative results are shown in FIG. **5**. The IC_{50} value for D2E7 inhibition of hTNFα-induced expression of ELAM-1 on HUVEC is approximately 6×10^{-11} M in these experiments. These results demonstrate that the D2E7 human anti-hTNFα antibody inhibits the hTNFα-induced expression of ELAM-1 on HUVEC at concentrations approximately equivalent to that of the murine anti-hTNFα mAb MAK 195.

In another series of experiments, the ability of the IgG1 form of D2E7 to inhibit hTNFα-induced expression of ELAM-1 on HUVEC was examined as described above. The results from three independent experiments, and the average thereof, are summarized below in Table 11:

TABLE 11

Inhibition of TNFα-Induced ELAM-1 Expression by D2E7 IgG1 Receptor

Experiment	IC_{50} [M]
1	1.95×10^{-10}
2	1.69×10^{-10}
3	1.90×10^{-10}
Average	$1.85\pm0.14\times10^{-10}$

This series of experiments confirmed that D2E7, in the full-length IgG1 form, inhibits TNFα-induced ELAM-1 expression on HUVEC with an average IC_{50} [M] of $1.85\pm0.14\times10^{-10}$.

The neutralization potency of D2E7 IgG1 was also examined for the rhTNF induced expression of two other adhesion molecules, ICAM-1 and VCAM-1. Since the rhTNF titration curve for ICAM-1 expression at 16 hours was very similar to the curve of ELAM-1 expression, the same concentration of rhTNF was used in the antibody neutralization experiments. The HUVEC were incubated with rhTNF in the presence of varying concentrations of D2E7 in a 37° C. CO_2 incubator for 16 hours, and the ICAM-1 expression was measured by mouse anti-ICAM-1 antibody followed by ^{125}I-labeled sheep anti-mouse antibody. Two independent experiments were performed and the IC_{50} values were calculated. An unrelated human IgG1 antibody did not inhibit the ICAM-1 expression.

The experimental procedure to test inhibition of VCAM-1 expression was the same as the procedure for ELAM-1 expression, except anti-VCAM-1 MAb was used instead of anti-ELAM-1 MAb. Three independent experiments were performed and the IC_{50} values were calculated. An unrelated human IgG1 antibody did not inhibit VCAM-1 expression.

The results are summarized below in Table 12:

TABLE 12

Inhibition of ICAM-1 and VCAM-1 Expression byD2E7 IgG1

ICAM-1 Inhibition		IC_{50} [M]	
Experiment	IC_{50} [M]	Experiment	IC_{50} [M]
1	1.84×10^{-10}	1	1.03×10^{-10}
2	2.49×10^{-10}	2	9.26×10^{-11}
		3	1.06×10^{-10}
Average	$2.17\pm0.46\times10^{-10}$	Average	$1.01\pm0.01\times10^{-10}$

These experiments demonstrate that treatment of primary human umbilical vein endothelial cells with rhTNF led to optimum expression of adhesion molecules: ELAM-1 and VCAM-1 at four hours, and the maximum up-regulated expression of ICAM-1 at 16 hours. D2E7 was able to inhibit the expression of the three adhesion molecules in a dose dependent manner. The IC_{50} values for the inhibition of ELAM-1, ICAM-1 and VCAM-1 were 1.85×10^{-10}, 2.17×10^{-10} and 1.01×10^{-10} M, respectively. These values are very similar, indicating similar requirements for the dose of rhTNF activation signal to induce ELAM-1, ICAM-1 and VCAM-1 expression. Interestingly, D2E7 was similarly effective in the longer inhibition assay of the the ICAM-1 expression. The ICAM-1 inhibition assay required 16 hours of co-incubation of rhTNF and D2E7 with HUVEC as opposed to 4 hours required for the ELAM-1 and the VCAM-1 inhibition assays. Since D2E7 has a slow off-rate for rhTNF, it is conceivable that during the 16 hour co-incubation period there was no significant competition by the TNF receptors on the HUVEC.

D. In Vivo Neutralization of hTNFαZ

Three different in vivo systems were used to demonstrate that D2E7 is effective at inhibiting hTNFα activity in vivo.

I. Inhibition of TNF-Induced Lethality in D-Galactosamine-Sensitized Mice

Injection of recombinant human TNFα (rhTNFα) to D-galactosamine sensitized mice causes lethality within a 24 hour time period. TNFα neutralizing agents have been shown to prevent lethality in this model. To examine the ability of human anti-hTNFα antibodies to neutralize hTNFα in vivo in this model, C57B1/6 mice were injected with varying con-

centrations of D2E7-IgG1, or a control protein, in PBS intraperitoneally (i.p.). Mice were challenged 30 minutes later with 1 μg of rhTNFα and 20 mg of D-galactosamine in PBS i.p., and observed 24 hours later. These amount of rhTNFα and D-galactosamine were previously determined to achieve 80-90% lethality in these mice.

Representative results, depicted as a bar graph of % survival versus antibody concentration, are shown in FIG. 6. The black bars represent D2E7, whereas the hatched bars represent MAK 195. Injection of 2.5-25 μg of D2E7 antibody per mouse protected the animals from TNFα-induced lethality. The ED_{50} value is approximately 1-2.5 μg/mouse. The positive control antibody, MAK 195, was similar in its protective ability. Injection of D2E7 in the absence of rhTNFα did not have any detrimental effect on the mice. Injection of a non-specific human IgG1 antibody did not offer any protection from TNFα-induced lethality.

In a second experiment, forty-nine mice were divided into 7 equal groups. Each group received varying doses of D2E7 thirty minutes prior to receiving an LD_{80} dose of rhTNF/D-galactosamine mixture (1.0 μg rhTNF and 20 mg D-galactosamine per mouse). Control group 7 received normal human IgG1 kappa antibody at 25 μg/mouse dose. The mice were examined 24 hours later. Survival for each group is summarized below in Table 13.

TABLE 13

24 Hour Survival After Treatment with D2E7

Group	Survival (alive/total)	Survival (%)
1 (no antibody)	0/7	0
2 (1 μg)	1/7	14
3 (2.6 μg)	5/7	71
4 (5.2 μg)	6/7	86
5 (26 μg)	6/7	86
6 (26 μg; no rhTNF)	7/7	100
7 (25 μg Hu IgG1)	1/7	14

II. Inhibition of TNF-Induced Rabbit Pyrexia

The efficacy of D2E7 in inhibiting rhTNF-induced pyrexia response in rabbits was examined. Groups of three NZW female rabbits weighing approximately 2.5 kg each were injected intravenously with D2E7, rhTNF, and immune complexes of D2E7 and rhTNF. Rectal temperatures were measured by thermistor probes on a Kaye thermal recorder every minute for approximately 4 hours. Recombinant human TNF in saline, injected at 5 μg/kg, elicted a rise in temperature greater than 0.4° C. at approximately 45 minutes after injection. The antibody preparation by itself, in saline at a dose of 138 μg/kg, did not elicit a rise in temperature in the rabbits up to 140 minutes after administration. In all further experiments, D2E7 or control reagents (human IgG1 or a saline vehicle) were injected i.v. into rabbits followed 15 minutes later by an injection of rhTNF in saline at 5 μg/kg i.v. Representative results of several experiments are summarized below in Table 14:

TABLE 14

Inhibition of rhTNF-induced Pyrexia with D2E7 in Rabbits

D2E7 dose (μg/kg)	Temp. rise*, ° C. rhTNF	rhTNF + D2E7	% Inhib.**	Molar Ratio D2E7:rhTNF	Peak Temp. minutes post rhTNF
14	0.53	0.25	53	1	60
24	0.43	0.13	70	1.6	40
48	0.53	0.03	94	3.3	50
137	0.53	0.00	100	9.5	60
792	0.80	0.00	100	55	60

*= Peak temperature

**= % inhibition = (1 − {temperature rise with rhTNF & D2E7/temperature rise with rhTNF alone}) × 100.

Intravenous pretreatment with D2E7 at a dose of 14 μg/kg partially inhibited the pyrogenic response, compared to rabbits pre-treated with saline alone. D2E7 administered at 137 μg/kg totally suppressed the pyrogenic response of rhTNF in the same experiment. In a second experiment, D2E7 administered at 24 μg/kg also partially suppressed the pyrogenic response, compared to rabbits pretreated with saline alone. The molar ratio of D2E7 to rhTNF was 1/6:1 in this experiment. In a third experiment, D2E7 injected i.v. at 48 μg/kg (molar ratio D2E7:rhTNF=3.3:1) totally suppressed the pyrogenic response, compared to rabbits pretreated with the control human IgG1 in saline at 30 μg/kg. In the final experiment, rabbits pretreated with D2E7 (792 μg/kg) at a very high molar ratio to rhTNF (55:1) did not develop any rise in temperature at any time up to 4 hours of observation. Treatment of rabbits with immune complexes generated from a mixture of D2E7 and rhTNF incubated at 37° C. for 1 hour at a molar ratio of 55:1, without subsequent rhTNF administration, also did not elicit any rise in temperature in the same experiment.

III. Prevention of Polyarthritis in Tg197 Transgenic Mice

The effect of D2E7 on disease development was investigated in a transgenic murine model of arthritis. Transgenic mice (Tg197) have been generated that express human wild type TNF (modified in the 3' region beyond the coding sequences) and these mice develop chronic polyarthritis with 100% incidence at 4-7 weeks of age (see *EMBO J.* (1991) 10:4025-4031 for further description of the Tg197 model of polyarthritis).

Transgenic animals were identified by PCR at 3 days of age Litters of transgenic mice were divided into six groups. Transgenic mice were verified by slot-blot hybridization analysis at 15 days of age. The treatment protocols for the six groups were as follows: Group 1=no treatment; Group 2=saline (vehicle); Group 3=D2E7 at 1.5 μg/g; Group 4=D2E7 at 15 μg/g; Group 5=D2E7 at 30 μg/g; and Group 6=IgG1 isotype control at 30 μg/g. A litter with non transgenic mice was also included in the study to serve as a control (Group 7 nontransgenic; no treatment). Each group received three i.p. injections per week of the indicated treatments. Injections continued for 10 weeks. Each week, macroscopic changes in joint morphology were recorded for each animal. At 10 weeks, all mice were sacrificed and mouse tissue was collected in formalin. Microscopic examination of the tissue was performed.

Animal weight in grams was taken for each mouse at the start of each week. At the same time measurements of joint size (in mm) were also taken, as a measurement of disease severity. Joint size was established as an average of three measurements on the hind right ankle using a micrometer device. Arthritic scores were recorded weekly as follows: 0=No arthritis, (normal appearance and flexion); +=mild arthritis Joint distortion); ++=moderate arthritis (swelling,

joint deformation) and +++=heavy arthritis (ankylosis detected on flexion and severely impaired movement). Histopathological scoring based on haematoxylin/eosin staining of joint sections was based as follows; 0=No detectable disease; 1=proliferation of the synovial membrane; 2=heavy synovial thickening 3=cartilage destruction and bone erosion.

The effect of D2E7 treatment on the mean joint size of the Tg197 transgenic arthritic mice is shown in the graph of FIG. 9. The histopathological and arthritic scores of the Tg197 transgenic mice, at 11 weeks of age, are summarized below in Table 15:

TABLE 15

Effect of D2E7 on Histopathology and Arthritic Score in Tg197 Mice

Group	Treatment	Histopathological Score	Arthritic Score
1	none	3 (7/7)	+++ (7/7)
2	saline	3 (8/8)	+++ (8/8)
6	IgG1 control	3 (9/9)	+++ (7/9)
3	D2E7 at 1.5 μg/g	0 (6/8)	0 (8/8)
4	D2E7 at 15 μg/g	0 (7/8)	0 (8/8)
5	D2E7 at 30 μg/g	0 (8/8)	0 (8/8)

This experiment demonstrated that the D2E7 antibody has a definite beneficial effect on transgenic mice expressing the wild-type human TNF (Tg197) with no arthritis after the study period.

D2E7 Neutralization of TNFαs from Other Species

The binding specificity of D2E7 was examined by measuring its ability to neutralize tumor necrosis factors from various primate species and from mouse, using an L929 cytotoxicity assay (as described in Example 4, subsection A, above). The results are summarized in Table 16 below:

TABLE 16

Ability of D2E7 to Neutralize TNF from Different Species in the L929 Assay

TNFα*	Source	IC_{50} for D2E7 Neutralization (M)**
Human	Recombinant	7.8×10^{-11}
Chimpanzee	LPS-stimulated PBMC	5.5×10^{-11}
baboon	Recombinant	6.0×10^{-11}
marmoset	LPS-stimulated PBMC	4.0×10^{-10}
cynomolgus	LPS-stimulated PBMC	8.0×10^{-11}
rhesus	LPS-stimulated PBMC	3.0×10^{-11}
canine	LPS-stimulated WBC	2.2×10^{-10}
porcine	Recombinant	1.0×10^{-7}
murine	Recombinant	$>1.0 \times 10^{-7}$

The results in Table 16 demonstrate that D2E7 can neutralize the activity of five primate TNFαs approximately equivalently to human TNFα and, moreover, can neutralize the activity of canine TNFα (about ten-fold less well than human TNFα) and porcine and mouse TNFα (about ~1000-fold less well than human TNFα). Moreover, the binding of D2E7 to solution phase rhTNFα was not inhibited by other cytokines, such as lymphotoxin (TNFβ), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IFNγ and TGFβ, indicating that D2E7 is very specific for its ligand TNFα.

F. Lack of Cytokine Release by Human Whole Blood Incubated with D2E7

In this example, the ability of D2E7 to induce, by itself, normal human blood cells to secrete cytokines or shed cell surface molecules was examined. D2E7 was incubated with diluted whole blood from three different normal donors at varying concentrations for 24 hours. An LPS positive control was run at the same time, at a concentration previously determined to stimulate immunocompetent blood cells to secrete cytokines. The supernatants were harvested and tested in a panel of ten soluble cytokine, receptor and adhesion molecule ELISA kits: IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNFα, soluble TNF receptor I, soluble TNF receptor II, soluble ICAM-1 and soluble E-selectin. No significant amounts of cytokines or shed cell surface molecules were measured as a result of D2E7 antibody co-incubation, at concentrations up to 343 μg/ml. Control cultures without the addition of the antibody also did not yield any measurable amounts of cytokines, whereas the LPS co-culture control yielded elevated values in the high picogram to low nanogram range. These results indicate that D2E7 did not induce whole blood cells to secrete cytokines or shed cell surface proteins above normal levels in ex vivo cultures.

Forming part of the present disclosure is the appended Sequence Listing, the contents of which are summarized in the table below:

SEQ ID NO:	ANTIBODY CHAIN	REGION	SEQUENCE TYPE
1	D2E7	VL	amino acid
2	D2E7	VH	amino acid
3	D2E7	VL CDR3	amino acid
4	D2E7	VH CDR3	amino acid
5	D2E7	VL CDR2	amino acid
6	D2E7	VH CDR2	amino acid
7	D2E7	VL CDR1	amino acid
8	D2E7	VH CDR1	amino acid
9	2SD4	VL	amino acid
10	2SD4	VH	amino acid
11	2SD4	VL CDR3	amino acid
12	EP B12	VL CDR3	amino acid
13	VL10E4	VL CDR3	amino acid
14	VL100A9	VL CDR3	amino acid
15	VLL100D2	VL CDR3	amino acid
16	VLL0F4	VL CDR3	amino acid
17	LOE5	VL CDR3	amino acid
18	VLLOG7	VL CDR3	amino acid
19	VLLOG9	VL CDR3	amino acid
20	VLLOH1	VL CDR3	amino acid
21	VLLOH10	VL CDR3	amino acid
22	VL1B7	VL CDR3	amino acid
23	VL1C1	VL CDR3	amino acid
24	VL0.1F4	VL CDR3	amino acid
25	VL0.1H8	VL CDR3	amino acid
26	LOE7.A	VL CDR3	amino acid
27	2SD4	VH CDR3	amino acid
28	VH1B11	VH CDR3	amino acid
29	VH1D8	VH CDR3	amino acid
30	VH1A11	VH CDR3	amino acid
31	VH1B12	VH CDR3	amino acid
32	VH1E4	VH CDR3	amino acid
33	VH1F6	VH CDR3	amino acid
34	3C-H2	VH CDR3	amino acid
35	VH1-D2.N	VH CDR3	amino acid
36	D2E7	VL	nucleic acid
37	D2E7	VH	nucleic acid

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

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<220> FEATURE:
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20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Arg Tyr Asn Arg Ala Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

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<223> OTHER INFORMATION: mutated human antibody

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20 25 30

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35 40 45

Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val
50 55 60

Glu Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Ser Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Lys Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

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<223> OTHER INFORMATION: Xaa = Tyr or Asn

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<211> LENGTH: 17
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Gly

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<210> SEQ ID NO 8
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<210> SEQ ID NO 9
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<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 9

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1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Arg Asn Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ala Ala Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Val Ala Thr Tyr Tyr Cys Gln Lys Tyr Asn Ser Ala Pro Tyr
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100 105

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<220> FEATURE:
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<400> SEQUENCE: 10

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1 5 10 15

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20 25 30

Ala Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Asp Trp Val
35 40 45

Ser Ala Ile Thr Trp Asn Ser Gly His Ile Asp Tyr Ala Asp Ser Val
50 55 60

Glu Gly Arg Phe Ala Val Ser Arg Asp Asn Ala Lys Asn Ala Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Thr Lys Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn Trp Gly
100 105 110

Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 11
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 11

Gln Lys Tyr Asn Ser Ala Pro Tyr Ala
1 5

<210> SEQ ID NO 12

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<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 12

Gln Lys Tyr Asn Arg Ala Pro Tyr Ala
1 5

<210> SEQ ID NO 13
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 13

Gln Lys Tyr Gln Arg Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 14
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 14

Gln Lys Tyr Ser Ser Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 15

Gln Lys Tyr Asn Ser Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 16
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 16

Gln Lys Tyr Asn Arg Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 17
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 17

Gln Lys Tyr Asn Ser Ala Pro Tyr Tyr
1 5

<210> SEQ ID NO 18
<211> LENGTH: 9

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 18

Gln Lys Tyr Asn Ser Ala Pro Tyr Asn
1 5

<210> SEQ ID NO 19
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 19

Gln Lys Tyr Thr Ser Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 20
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 20

Gln Lys Tyr Asn Arg Ala Pro Tyr Asn
1 5

<210> SEQ ID NO 21
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 21

Gln Lys Tyr Asn Ser Ala Ala Tyr Ser
1 5

<210> SEQ ID NO 22
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 22

Gln Gln Tyr Asn Ser Ala Pro Asp Thr
1 5

<210> SEQ ID NO 23
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 23

Gln Lys Tyr Asn Ser Asp Pro Tyr Thr
1 5

<210> SEQ ID NO 24
<211> LENGTH: 9
<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 24

Gln Lys Tyr Ile Ser Ala Pro Tyr Thr
1 5

<210> SEQ ID NO 25
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 25

Gln Lys Tyr Asn Arg Pro Pro Tyr Thr
1 5

<210> SEQ ID NO 26
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 26

Gln Arg Tyr Asn Arg Ala Pro Tyr Ala
1 5

<210> SEQ ID NO 27
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 27

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asn
1 5 10

<210> SEQ ID NO 28
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 28

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Lys
1 5 10

<210> SEQ ID NO 29
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 29

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Tyr
1 5 10

<210> SEQ ID NO 30
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 30

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu Asp Asp
1 5 10

<210> SEQ ID NO 31
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 31

Ala Ser Tyr Leu Ser Thr Ser Phe Ser Leu Asp Tyr
1 5 10

<210> SEQ ID NO 32
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 32

Ala Ser Tyr Leu Ser Thr Ser Ser Ser Leu His Tyr
1 5 10

<210> SEQ ID NO 33
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 33

Ala Ser Phe Leu Ser Thr Ser Ser Ser Leu Glu Tyr
1 5 10

<210> SEQ ID NO 34
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 34

Ala Ser Tyr Leu Ser Thr Ala Ser Ser Leu Glu Tyr
1 5 10

<210> SEQ ID NO 35
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 35

Val Ser Tyr Leu Ser Thr Ala Ser Ser Leu Asp Asn
1 5 10

<210> SEQ ID NO 36
<211> LENGTH: 321
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 36

gacatccaga tgacccagtc tccatcctcc ctgtctgcat ctgtagggga cagagtcacc 60

atcacttgtc gggcaagtca gggcatcaga aattacttag cctggtatca gcaaaaacca 120

gggaaagccc ctaagctcct gatctatgct gcatccactt tgcaatcagg ggtcccatct 180

cggttcagtg gcagtggatc tgggacagat ttcactctca ccatcagcag cctacagcct 240

gaagatgttg caacttatta ctgtcaaagg tataaccgtg caccgtatac ttttggccag 300

gggaccaagg tggaaatcaa a 321

<210> SEQ ID NO 37
<211> LENGTH: 363
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: mutated human antibody

<400> SEQUENCE: 37

gaggtgcagc tggtggagtc tgggggaggc ttggtacagc ccggcaggtc cctgagactc 60

tcctgtgcgg cctctggatt cacctttgat gattatgcca tgcactgggt ccggcaagct 120

ccagggaagg gcctggaatg ggtctcagct atcacttgga atagtggtca catagactat 180

gcggactctg tggagggccg attcaccatc tccagagaca acgccaagaa ctccctgtat 240

ctgcaaatga acagtctgag agctgaggat acggccgtat attactgtgc gaaagtctcg 300

taccttagca ccgcgtcctc ccttgactat tggggccaag gtaccctggt caccgtctcg 360

agt 363

The invention claimed is:

1. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate, such that the rheumatoid arthritis is treated, wherein the antibody is an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-7} M or less, and neutralizes TNFα-induced cellular activation in a standard in vitro assay for TNRα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC).

2. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of 5×10^{-4} s^{-1} or less.

3. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of $1\times10^{-5}s^{-1}$ or less.

4. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-8} M or less.

5. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-9} M or less.

6. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard in vitro L929 assay with an IC_{50} of 1×10^{-10} M or less.

7. The method of claim 1, wherein the isolated human antibody, or antigen-binding portion thereof, is a recombinant antibody, or antigen-binding portion thereof.

8. A method for treating a subject suffering from rheumatoid arthritis, comprising administering to the subject both an antibody and methotrexate, such that the rheumatoid arthritis is treated, wherein the antibody is an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes TNFα-induced cellular activation in a standard in vitro assay for TNFα-induced ELAM-1 expression on human umbilical vein endothelial cells (HUVEC).

9. The method of claim 8, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of 5×10^{-4} s^{-1} or less.

10. The method of claim 8, wherein the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} rate constant of 1×10^{-4} s^{-1} or less.

11. The method of claim 8, wherein the isolated human antibody, or antigen-binding portion thereof, is a recombinant antibody, or antigen-binding portion thereof.

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